The Cryopreservation of *Picea sitchensis* Germplasm

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This research programme was carried out in collaboration with the Northern Research Station of Forest Research (An agency for the UK Forestry Commission)

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed.....[redacted] **Date**...10/1/06.....
Director of Studies

Declaration

I hereby declare that the following thesis is based on the results of investigations conducted by myself and that this thesis is of my own composition. This thesis has not, in whole or part, been previously presented for a higher degree. Work other than my own is clearly indicated in the text by reference to the relevant researchers or their publications.

Signed: .....[redacted].

Samantha Gale

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Abbreviations[1]

A	
Abscisic acid	ABA
B	
6- Benzylaminopurine	BAP
C	
Cetryltrimethyl ammonium bromide	CTAB
The Conservation of a Vital European Scientific and Biotechnological Resource: MicroAlgae and Cyanobacteria	COBRA
Establishing Cryopreservation Methods for Conserving European Plant Germplasm	CRYMCEPT
D	
Degrees celsius	°C
Deoxyribonucleic acid	DNA
2,4-Dichlorophenoxyacetic acid	2,4-D
Dimethyl sulphoxide	DMSO
Dry weight	Dry weight
E	
Ethylene diamine tetra-acetic acid	EDTA
Embryogenic suspension	ES
Embryogenic suspensor mass	ESM
F	
Fluorescein diacetate	FDA
Fresh weight	FW
G	
Gas chromatography	GC
General linear model	GLM
Gibberelic acid	GA_3
H	
High Performance Liquid Chromatography	HPLC
Hour	hr
-4-Hydroxy-2-nonenal	HNE
Hydrochloric acid	HCL
Hydrogen peroxide	H_2O_2
Hydroxyl free radical	·OH
I	
Indole-3-acetic acid	IAA
J, K, L	
Liquid nitrogen	LN
Least significant difference	LSD
M	
Malondialdehyde	MDA
Moisture content	MC

[1] Detailed terminology explainations in the Glossary in Appendix

Methane	CH_4
Microgram	µg
Microlitre	µl
Millilitre	ml
Micromolar	mM
Minute	min
Murashige and Skoog (1962) Medium	MS
N	
Non embryogenic mass	NEM
Northern Research Station, Roslin	NRS
O	
Osmotically active water	OA
Osmotically inactive water	OI
P	
Percentage	%
Plant Vitrification Solution 2	PVS2
Plant growth regulator	PGR
Q, R	
Relative humidity	RH
Relative moisture	% RM
Ribonucleic acid	RNA
S	
Second	s
Standard error of the mean	SEM
Somatic Embryogenic ABA	SEABA
Somatic Embryogenic Maintenance Medium	SEMM
Somatic Embryogenic Initiation Medium	SEIM
Sucrose	suc
T	
Thidiazuron	TDZ
Tris-x-aminomethane	Tris
2,3,5-Triphenyltetrazolium chloride	TTC
Glass transition	Tg
U	
Units of activity	U
University of Abertay Dundee	UAD
V	
Volume/volume	v/v
W	
Weight/volume	w/v

Abstract

The Cryopreservation of *Picea sitchensis* Germplasm

Samantha Gale

Picea sitchensis is an important tree species for UK forestry and is at the forefront of prototype clonal breeding programs. These can only be implemented using *in vitro*, culture therefore cryopreservation technology development is imperative such that elite germplasm can be conserved, without compromise to genetic integrity, whilst phenotypic validation of selected genotypes is undertaken.

Three explant types were transferred from the Northern Research station, Roslin to the University of Abertay, Dundee where cryopreservation testing was initiated. Each explant comprised of different anatomical complexities varying from the simplest dedifferentiated embryogenic suspensor masses, to matured somatic embryos and whole tissue shoot-tip apices. Before cryopreservation was initiated *in vitro* cultures were stabilised and characterised at UAD. Shoot cultures showed different growth responses between genotypes and between culture locations, but biochemical profiling of oxidative stress markers, ethylene and DNA methylation did not confirm stress or epigenetic change as the cause of these differences and physiological recalcitrance.

A cryopreservation protocol, using a programmable freezer (Planar), was successfully developed and is reported for the first time for *P. sitchensis* embryogenic suspensor masses (ESM). Post-LN survival rates of up to 100% were observed in several genotypes. Encapsulation-dehydration was successfully utilised to cryopreserve *P. sitchensis* mature somatic embryos reported for the first time. Recovered embryos were able to re-initiate into dedifferentiated non-embryogenic masses (NEM) (up to 100%) and embryogenic suspensor masses (ESM) (up to 20% post –LN) as a source of material to mass multiply cryopreserved clonal offspring. Major steps were progressed for the most recalcitrant conifer explant in the project, shoot-tip apices, with essential pre-treatment steps established. Critical cryogenic factors were determined through thermal analysis using Differential Scanning Calorimetry.

The study concluded with the initiation of technology transfer of cryopreservation methods to the Northern Research Station to establish the UK's first conifer cryobank. These systems were preliminary validated. Further implementation will proceed out with the timescale of this project but based on recommendations generated from this thesis.

Chapter 1 GENERAL INTRODUCTION

1. Aims of the project

The aims of the project are to develop cryopreservation protocols for *in vitro* shoot-tips and somatic embryos, and to apply, validate and improve existing cryopreservation methods for the cryo-conservation of embryogenic suspensor masses of Sitka spruce. The research combines the study of applied clonal forestry, *in vitro* plant biology, cryopreservation and fundamental biochemistry and thermodynamics. It applies these investigations to assist in the establishment of a pilot cryopreservation genebank for *Picea sitchensis* (Sitka spruce) germplasm at the Northern Research Station for the UK Forestry Commission.

Specific aims include:

- To apply traditional and contemporary cryopreservation protocols to Sitka spruce explants; shoot-tip cultures, embryogenic suspensor masses and somatic embryos.
- To trial optimised protocols over three years and apply them to a broad range of genotypes.
- To investigate the use of cold acclimation, plant hormones and carbohydrates in cryopreservation pre-treatment and post-treatment in shoot-tip apices.
- To investigate *in vitro* stress and cryopreservation recalcitrance using free radical chemistry and thermal imaging analytical techniques.
- To initiate technology transfer to Northern Research Station and compile critical factors and recommendations for future applications of cryopreservation in the UK Forestry Commission.

1.1 Introduction

Clonal forestry comprises several stages involving the selection and propagation of elite trees with desirable traits (such as straightness, growth rate and timber density) (Samuel, *et al.*, 2000). Elite "Plus" trees are field selected and grafted onto seed orchard stock for controlled breeding. Conifer phenotype is influenced by complex interactions between genotype and environmental factors. Consequently, it is difficult to ensure that desired characteristics are manifested in offspring phenotypes and even when the process is aided by the use of molecular markers it is not infallible. Thus, phenotype validation also requires the maturation of seedlings (6 years old) to determine the presence of desired characteristics. The biotechnological production of elite clonal material is an important adjunct to contemporary forestry as it allows the maintenance of known phenotypical traits. This approach is

dependent upon establishing morphogenically competent cultures *in vitro,* for which there are two developmental options: shoot or somatic embryogenic cultures. However, the successful initiation of plant tissue cultures is dependent upon the use of explants that are in a suitably juvenile state to ensure morphogenic competency. Achieving this is problematic for perennial woody plant species as the acquisition of juvenile tissue is compromised by the need to ascertain desirable trait expression in the mature condition.

One option is to maintain *in vitro* cultures from juvenile explants whilst the progeny are tested. Somatic embryogenesis, first developed by Hakman and von Arnold, (1985), is the most effective *in vitro* system to date for conifers. The process involves the initiation and culture of immature or mature zygotic embryos in culture medium containing optimised levels of plant growth regulators, namely 2,4-dichlorophenoxyacetic acid, (2,4-D) (John, *et al.,* 1995). The subsequent heterogeneous mix of cell clusters can be maintained either as a solid mass, embryogenic suspensor masses (ESM) or in a liquid, as embryogenic suspension (ES) dependent upon operating facilities and user preferences. Both cultures are maintained by regular sub-culturing or can be matured, by altering the growth regulator composition, to stimulate differentiation into somatic embryos and emblings (an intermediate stage between a fully developed somatic embryo and plantlet).

The maintenance of *in vitro* cultures also poses some specific problems as morphogenic competence (and thus ability to regenerate whole plants) declines over time in culture. The process is costly with respect to staffing operations and resources, and genetic and epigenetic change can increase with time in culture (Scowcroft, 1984). For these reasons cryopreservation has been integrated with *in vitro* conifer clonal forestry practises. Newly initiated somatic embryogenic cultures are placed in cryogenic storage where they can be maintained during Plus tree testing. This approach has added advantages in that problems associated with genetic instability are obviated (Fourre, *et al.,* 1997), it is less costly and time- and resource- intensive compared with keeping cultures in an actively growing state. Investigations into rice cell cryopreservation (Lynch, *et al.,* 1994) have indicated that *in vitro* ageing of cultures and associated cellular attributes (vacuole size) and free radical mediated stress (Benson, 1990) may influence cryotolerance. To date, there are no reports as to how prolonged *in vitro* culture may affect cryotolerance in conifers.

The development of cryopreservation for conifer cultures has progressed using methods originally developed for non-woody plant cells and tissues. (Kartha, 1987, Withers, 1983). These involve minimizing intracellular ice formation through pre-growth osmotic dehydration treatments followed by colligative cryoprotection and controlled rate (programmable

freezing) prior to liquid nitrogen immersion. A number of conifer-specific cryopreservation methodologies, using step-wise addition of cryoprotectants, have been optimised for ESM, *Picea abies* and loblolly pine (Galerne and Dereuddre, 1988, Gupta, *et al.*, 1987) and for ES: *Picea sitchensis* (Find, *et al.*, 1993); *Picea mariana* and *Larix* x *eurolepis* (Klmaszewska, *et al.*, 1992), and *Picea glauca* (Find, *et al.*, 1993, Kartha, *et al.*, 1988). A simplified freezing method for ES using isopropanol filled polycarbonate units (Mr. Frosty ™, Nalgene ® Tyne and Wear, England) in a -80°C freezer has been reported (Cyr, *et al.*, 1994). A PVS2 based vitrification method has also been applied to *P. mariana* (Touchell, *et al.*, 2002).

To develop cryopreservation protocols that meet the needs of large-scale clonal forestry operations cryostorage methods must be applicable to a diverse range of unrelated genotypes. Whilst this has been achieved to some extent (Cyr, *et al.*, 1994, Park, *et al.*, 1993) for certain species, this is not the case in other species, an example being the somatic embryogenic cultures of Sitka spruce.

1.2 Clonal Forestry in the UK

Trees in Britain show large differences in growth rate and form throughout their distribution. To utilize traits desirable for commercial forestry the inheritance of traits needed to be better understood. Breeding work was initiated by the UK Forestry Commission in the 1920s, to establish if the superiority of an individual is passed on through seed to its progeny in the next generation. The basic hereditary principles of this work were derived from the investigations of Gregor Mendel's 1866 reseach, re-discovered in the 1900s (Hartmann and Kester, 2002).

The first form of tree improvement was to use superior stands of trees (Samuel, *et al.*, 2000). Well-adapted seed was collected but the progeny were not associated with high levels of improvement. Heritability estimates of early growth from population studies suggest that the environment would be expected to influence the phenotype of a tree six times more than the genotype (Hartmann and Kester, 2002). Higher gains were achieved by concentrating selection to superior individuals, "Plus trees"[2].

Progeny testing, to determine whether the superiority of a Plus-tree is inherited by its progeny, was undertaken by crossing known Plus trees (using controlled pollination) and growing them at different sites. The progeny tree must be of a certain age or maturity (usually over 5 years) to show whether or not it has inherited parental Plus tree traits. Once the breeder

[2] Clarification of specialist terminology in the Glossary located in the Appendix

has identified the true superior Plus trees, grafts can be taken and planted in a clonal seed orchard, where the genetically superior progeny will be reproduced and collected as commercial quality seed (Ahuja and Libby, 1993). This procedure, from Plus tree selection to the production of commercial seed from an orchard, can take at least 20 years.

This process can be accelerated by creating clone banks of untested cuttings at the same time as progeny testing. However, cuttings have two major drawbacks: (1) they are expensive to produce, and the cost may outweigh the genetic gain to the buyer and (2) they can only be easily be propagated from juvenile (<8yrs old trees); older trees derived from cuttings show problems in rooting and grow plagiotrophically [a horizontal branchlike growth that is not desirable in forestry terms (Hartmann and Kester, 2002)]. If tested clones were to be used for afforestation, techniques would need to be developed that would keep a sample of the genotype in a juvenile form whilst the genotype is tested. *In vitro* techniques were the most promising methods by which this could be achieved.

1.2.1 Breeding history of *Picea sitchensis* (Sitka spruce)

Sitka spruce [*Picea sitchensis* (Bong.) Carr.] is the most important tree species in upland Britain and comprises over 50% of current planting (Rook, 1992). David Douglas introduced Sitka spruce to Britain in 1831, from the west coast fog-belt of North America where it is native. Douglas noted, "It has the great advantage that it thrives on poor soils, and could become a large and useful tree in Great Britain."

Holmes, (1987) described the Sitka spruce's unique combination of qualities including its:

(1) adaptability – it thrives well in the British oceanic climate and can tolerate high climatic exposure and a variety of soil conditions;
(2) form – shows strong apical dominance producing good straight stem form;
(3) timber quality – very versatile soft-wood timber especially suitable for paper pulp; and
(4) vigour – due to photosynthetic efficiency it is reported to be one of the fastest growing species of tree in the UK.

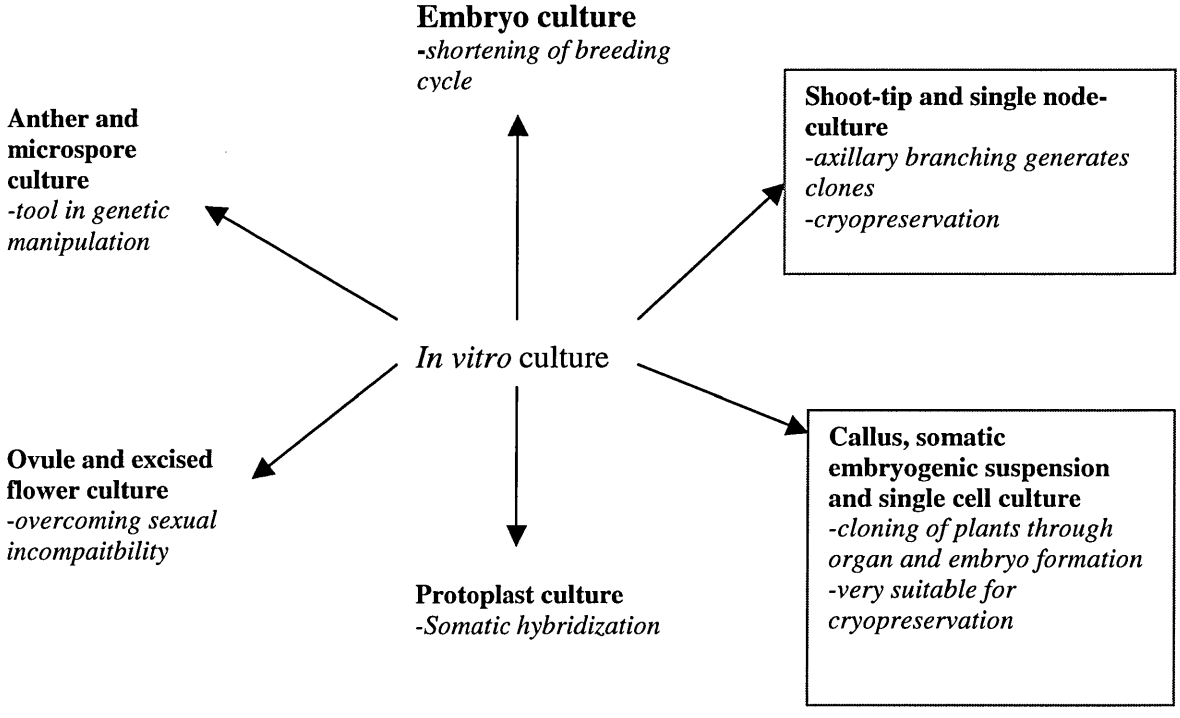
A tree breeding programme for Sitka spruce was initiated in the early 1960s by the UK Forestry Commission, at a time when the need in Britain was to establish timber on land that was poor for arable farming. Material from the Queen Charlotte Islands was the best adapted for use in Britain (Hibberd, 1991). The superior genotypes that have been identified offer appreciable genetic gains over unimproved material. Successful propagation techniques using stem cuttings were established, but due to the aforementioned problems with cuttings another propagation technique was required.

1.3 *In vitro* production for clonal forestry

1.3.1 Plant tissue culture history

The foundation of plant cell and tissue culture was based on the 'totipotency theory' postulated by Scwann and Schleiden in 1838, stating that plant cells were autonomic and capable of regenerating a complete plant (Pierik, 1987). The first attempt at plant tissue culture, by Haberlandt in 1902, failed (Pierik, 1987). In 1939, Gautheret achieved the first real plant tissue culture of continuously growing carrot callus (Pierik, 1987). The combination of increased post 2nd World War development in this field, for agriculture, forestry and horticulture, and the discovery of plant growth regulators such as the auxin (indole-3-acetic acid, IAA) and the cytokinin (kinetin) lead to rapid research development. In the 1960s the complete nutritional requirements for maintaining different types of plant cells were established. Soon after, MS medium (Murashige and Skoog, 1962) was formulated containing sucrose, basic salts, vitamin and plant growth regulators. MS medium forms the basis for routine subculture and development for many plant species today. Plant tissue culture has become a critical part of industry and research and it is an important biotechnological tool. A range of *in vitro* explants and tissue types can be formed, by varying environmental conditions and plant hormones, exploited and developed according to user requirements. Figure 1.1 shows a number of explant types and some of their biotechnological uses.

Figure 1.1 *In vitro* plant explant types and uses



1.3.2 Micropropagated plants

Micropropagation is the rapid vegetative propagation of plants *in vitro*. Plants can be cloned rapidly, without a full life cycle and propagation circumvents lengthy seed production or field-based vegetative reproduction, under highly controlled conditions. Micropropagated cultures can be generated from explants such as buds, stem nodal cuttings, or seed taken from parent plants. The explant is sterilised and placed in shoot proliferation medium. Once shoot-tips are established they can be maintained through routine subculture of the apical meristem or multiplied by single-node cuttings or axillary branching. A single node cutting gives rise to a shoot because there are vestigial nodes in the axils of the leaves and in the involucral bracts of the inflorescence, (Pierik, 1987). Micropropagation is important in plant breeding; genotypes can be maintained and used for seed production or rootstock and while clones can be generated for genebanking.

Whole plants were first derived from *in vitro* shoot-tips in *Lupinus* and *Tropaelum,* in 1946 by Ball (Pierik, 1987). Since then many culture systems have been established to maintain healthy plants in a disease and virus–free environment. The system is particularly amenable to species and cultivars that generate little seed such as in banana, fig, grape and petunias (Bhojwani and Razdan, 1983) and for vegetatively propagated plants *in vivo*.

There are many international culture collections based on shoot-tip cultures including: the International Potato Centre (CIP) in Peru, the National Bureau of Plant Genetic Resources (NBPGR) in India and the International Network for the Improvement of Banana and Plantain in Belgium. Shoot-tip cultures have also been utilised to conserve rare and threatened species such as *Primula scotica* (Benson, *et al.*, 2000) and medicinal plants such as *Rauvolfia serpentine* (Kataria and Shekhawat, 2005).

1.3.2.1 Micropropagation in trees and conifers

Since the 1930s, tree species have been amenable to various tissue culture techniques.
Woody species are more difficult to clone *in vitro* than herbaceous species because: (1) they have reduced totipotency largely due to maturation and dormancy issues, (2) research was initiated later, (3) the multiplication rate is lower, (4) dormancy plays a role (buds do not open and stem elongation fails), (5) surface sterilisation is more difficult, (6) genetic variation is greater in trees, (7) woody species are more liable to be affected by excretion of toxic substances such as phenolics into nutrient media, (8) trees can often only be selected for cloning when they are adult, and (9) explants are taken from field-grown plants, so there may

be considerable variation in explants due to different growth conditions and annual climate differences (Pierik, 1987). The first *in vitro* study in conifers, in the UK, was undertaken in 1974 at Leicester University (Samuel, *et al.,* 2000). This work on Sitka spruce and lodgepole pine demonstrated that adventitious buds could be induced on explants up to two years old.

Micropropagation of a number of conifer species has been achieved through the use of plant growth regulators to induce the formation of adventitious buds on isolated structures such as intact embryos in *Pinus contorta* (Von Arnold and Eriksson, 1981), cotyledons in Douglas fir (Cheng and Voqui, 1977), vegetative buds in *P. abies* (Von Arnold and Eriksson, 1979) and needles in *P. abies* (Jansson and Bornmann, 1980). *P. sitchensis* was first micropropagated by John and Murray (1981), without plant growth regulators because Sitka spruce formed axillary buds suitable for multiplication in the normal course of development.

1.3.3 Somatic embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells through a series of development similar to zygotic embryogenesis. This was first achieved in carrot in 1958 (Steward, *et al.,* 1958). Somatic embryogenesis does occur in nature, such as from the nucellar tissue in *Citrus*, but is more commonly a manifestation of the culture process. Somatic embryogenesis has now been reported for many plant species including woody angiosperms and gymnosperms. Embryogenesis can be induced either directly from an explant without a callus phase, or indirectly after a proliferation of callus or dedifferentiated mass (Gupta and Grob, 1995). Indirect embryogenesis is more common and explants are taken from plants or seed and placed on medium containing high concentrations of plant growth regulators (usually auxin such as 2,4-D) to achieve the rapid rate of cell division required for embryo initiation. Although abscisic acid (ABA) is required for the initiation of embryo maturation subsequent development is hormone autonomous and additional hormones usually disrupt embryo development (Warren, 1991). Embryos usually develop from surface cells of meristematic clusters in dedifferentiated masses, although it is thought that all cells in the cluster are competent to undergo embryogenesis. The embryos undergo a series of cellular and biochemical development stages from 'globular' to 'torpedo' to form 'emblings' (embryo derived seedlings).

Somatic embryogenesis offers an inexpensive method for large-scale propagation of selected genotypes. There are several production advantages: (1) a large number of plantlets are produced rapidly, (2) root and shoot meristem development occurs in the the same process, (3) it is amenable to cryopreservation and long-term storage (to be discussed in further detail),

(4) may be used for 'synthetic seed' as a means of and direct delivery of emblings facilitating automation. This would enable the production of seeds throughout the year and remove the uncertainty and risks of seed orchard production.

1.3.3.1 Somatic embryogenesis in conifers

Somatic embryogenesis has been very successful in conifers, largely because of the natural occurrence of simple polyembryony to induce the formation of genetically different embryos within the seed *in vivo,* and the *in vitro* induced and natural, cleavage polyembryony, whereby one embryo cleaves to form several genetically identical embryos (Gupta and Grob, 1995).

Somatic embryogenesis in conifers was first reported in 1985 from immature zygotic embryos of *P. abies* (Chalupa, 1985, Hakman and Arnold, 1985). Many researchers achieved successful re-generation of conifer plantlets via somatic embryogenesis. They termed the resultant culture embryonal or embryogenic suspensor masses due to its high degree of organisation. ESM of conifers is distinctly different to callus. ESM is described as white, translucent, moist or mucilaginous, consisting of anatomically early stage embryos which have an embryonal head and a suspensor system (Gupta, *et al.*, 1987). Non-embryogenic callus is described as opaque, friable (meaning as crumbs) and it greens in light with, or without, anatomical features. Somatic embryogenesis has been achieved in many genera of conifers including *Abies*, *Agathis*, *Larix, Picea, Pinus, Pseudotsuga* and *Sequoia.*

1.3.3.2 Somatic embryogenesis in *Picea sitchensis*

Somatic embryogenesis induction from immature embryos and resulting in soil established plantlet production has been reported in *P. sitchensis* (Krogstrup, 1988, Roberts, 1991). Significant research has been undertaken in *P. sitchensis* but there are still barriers to implementing a clonal forestry program (John, *et al.,* 1995). Genetic stability and field performance investigations still need to be undertaken. The establishment of ESM from mature tested *P. sitchensis* trees remains a problem. This may be circumvented through the cryopreservation of juvenile explant derived ESM or somatic embryos, that can be stored in liquid nitrogen (LN) during genotype testing and then thawed and deployed when superior clones are identified. Figure 1.2 shows how a clonal forestry propagation system for Sitka spruce might be developed. *In vitro* explant cultures of Sitka spruce embryogenic suspensor masses (ESM) (Fig. 1.2 -3a), somatic embryos (Fig. 1.2-3b) and shoot-tips (Fig. 1.2-3c) will be initiated and cryopreservation protocols developed so that plantlets can be readily matured once progeny testing has identified the superior trait genotypes.

1.3.4 Establishment of *Picea sitchensis* cultures

Families of shoot-tip cultures and embryogenic cultures (both ESM and somatic embryos) are initiated in different ways and derived from seeds that have undergone different pollination procedures. Figure 1.3 shows the half-sibling embryogenic culture genotypes produced by open pollination in a seed orchard. Figure 1.4 shows the full sibling shoot-tip culture genotypes produced by artificial pollination. It is possible to have half- and full-sibling families of both types of culture; culture determination in this project was by NRS choice. It is important to recognise the differences in genetic inheritance to interpret genotype and family differences to treatment responses and in progeny phenotype comparisons.

Figure 1.2 Clonal forestry programme for *Picea sitchensis*

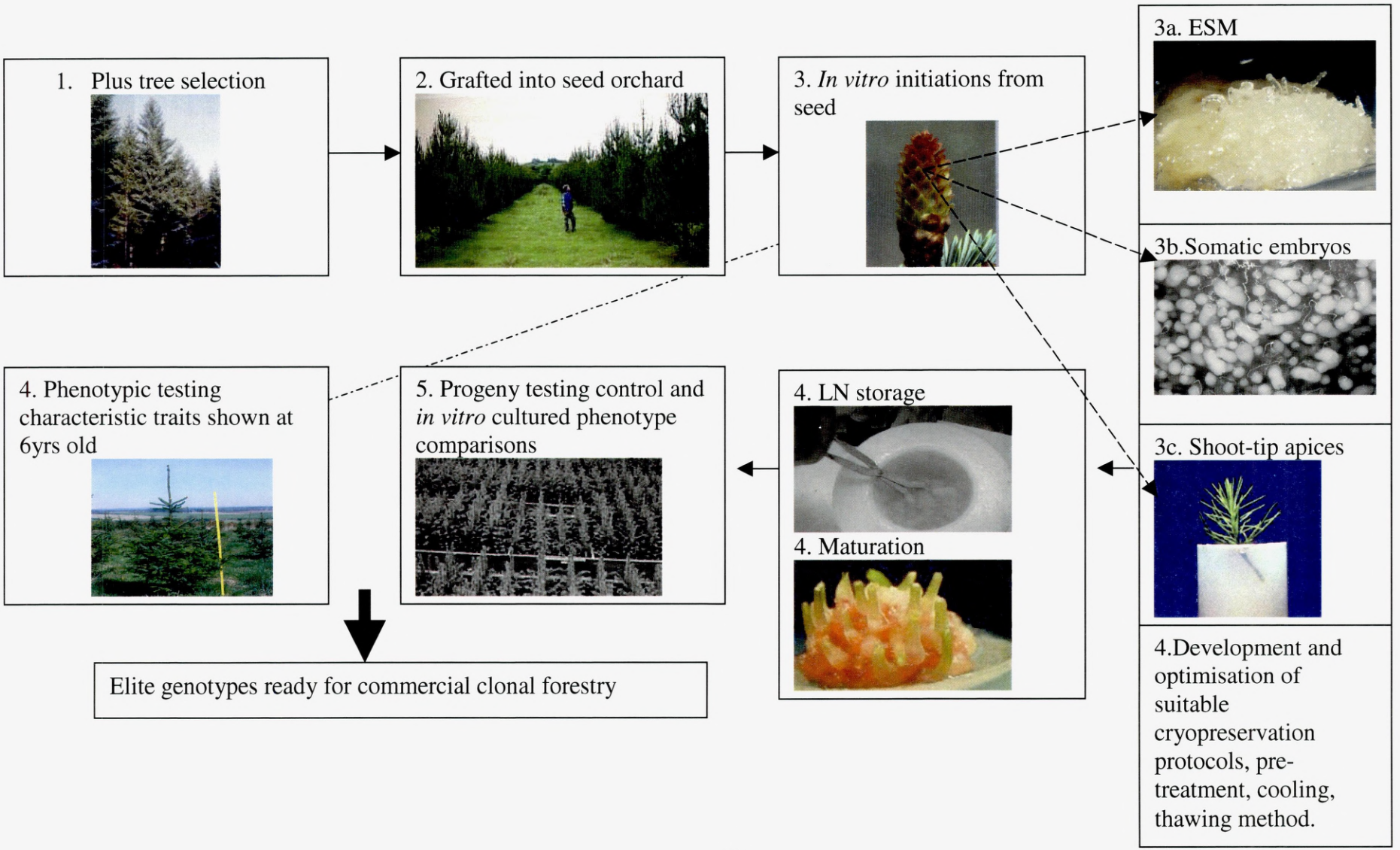


Figure 1.3 ESM origin and development

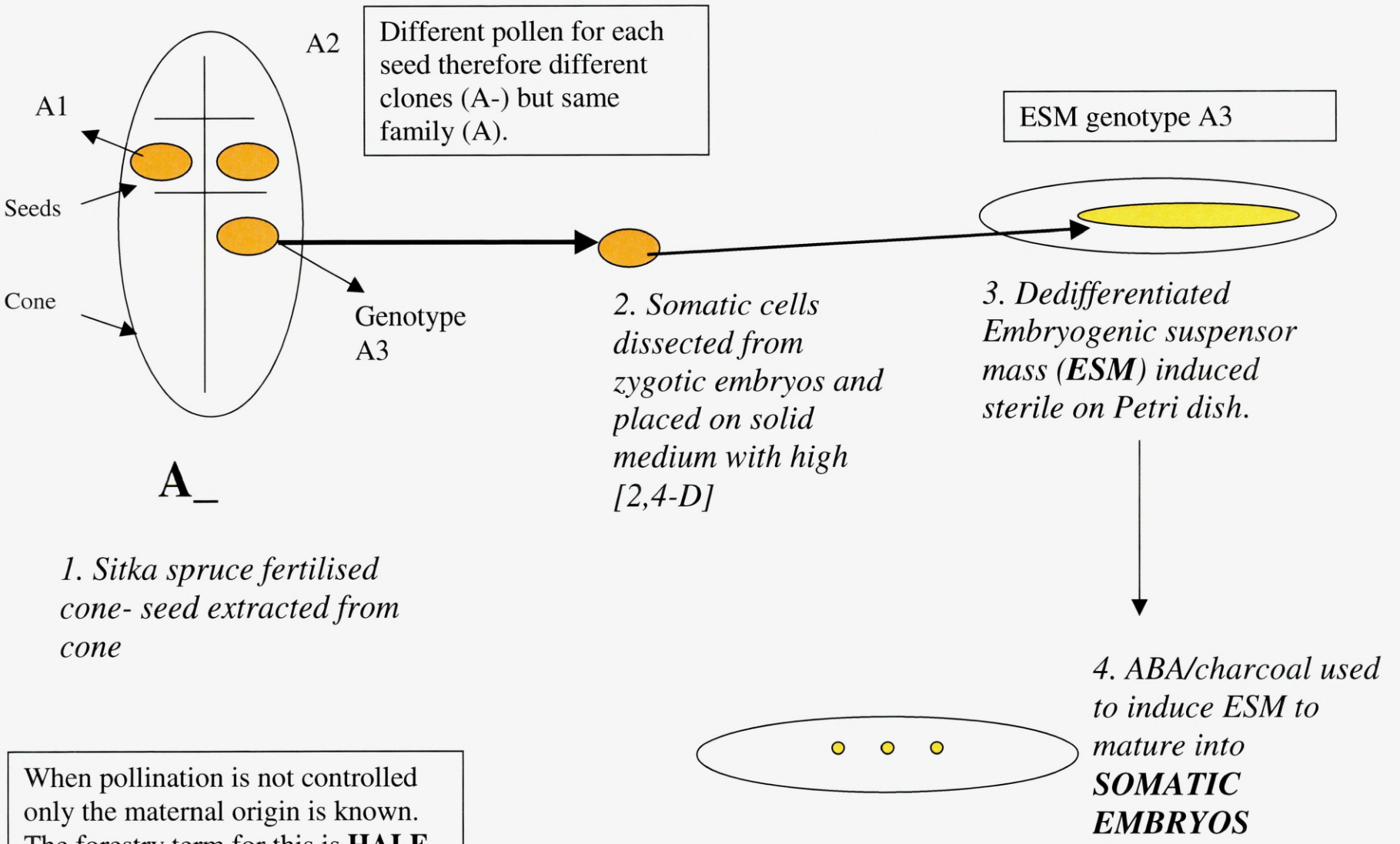
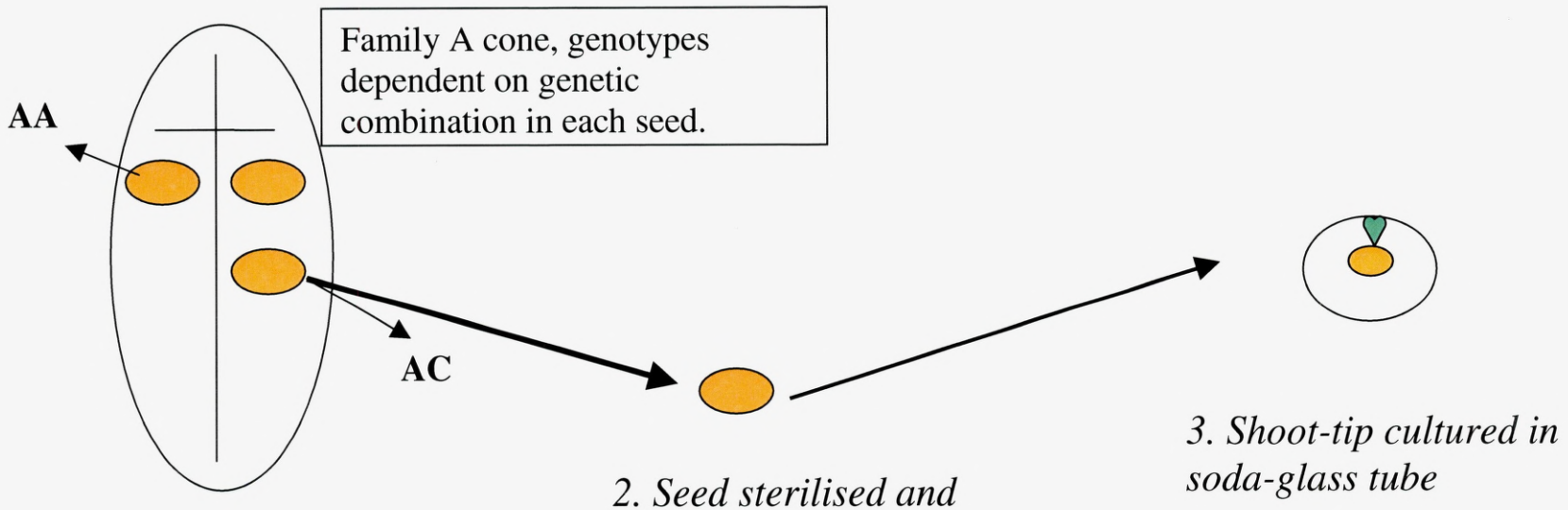


Figure 1.4 *In vitro* shoot-tip origin and development



Pollination controlled both maternal and paternal origin known- Forestry term **FULL-SIBLING**

1.3.5 *In vitro* stress: importance in woody plants

Woody plant species are often difficult to culture (Benson, 2000) and *in vitro* stress, ageing and recalcitrance play a significance role. Plant tissue recalcitrance has been defined as,'the inability of plant cells, tissues and organs to respond to tissue culture manipulations' (Benson, 2000). Investigations of *in vitro* stress are important in the context of this study because post-cryopreservation success may be compromised if physiologically stressed explants are incorporated in testing. Recalcitrance will be reviewed in the context of genetic predeterminism, gene expression patterns, whole and explant physiology, stress physiology, habituation and neoplastic progression. Each of these factors may have important impacts on *in vitro* conservation and pre-and post-cryopreservation manipulations and therefore they are considered as follows.

1.3.5.1 Genetic pre-determinism

In vivo woody plant life cycles.

Temperate woody perennial species have developed complex seasonal responses that enable them to survive winter extremes such as short day lengths, low light intensities and reduced temperatures. Such species have evolved the necessary cycles of dormancy, senescence, quiescence and rejuvenation that may be manifested as a whole plant response or through seed and vegetative bud cycles. Conifer species, in particular, show strong seasonal shoot growth variation, characterised by episodic flushes during the growing season.

In vivo life cycle impacts on in vitro performance

The seasonal growth dynamics in woody perennials may complicate control of plant cultures *in vitro* (Mccown, 2000). In woody shoot cultures, the 'stabilisation stage,' is considered the most difficult in *in vitro* shoot physiology. A 'stabilised shoot' culture is considered to be one that displays continuous and uniform growth as long as culture is maintained correctly and optimally sub-cultured (McCown, 2000). Unstable cultures show erratic growth, phenolic exudation and callusing.

The most successful trees to stabilise in micro-propagation show genetically determined continuous, non-episodic (sympodial shoot) growth, such as in poplars, birches and elms. Conifer species are particularly difficult to morphologically stabilise because the seasonal growth fluctuations (episodic flushes, monopodial shoot growth) are continued through *in vitro* culture. Seasonal patterns in *in vitro P. sitchensis* cultures have been observed (Selby and Harvey, 1985) depending on the time of year explants are procured. In the periods of

inactivation, cultures often deteriorate. These difficulties may be further elucidated in selected genotypes that may not be responsive to *in vitro* initiation through general initiation protocols.

1.3.5.2 Macro level –whole and explant physiology

There are several factors relating to the explant initiation and maintenance that may influence the *in vitro* culture success. The health and juvenility of the explant will determine whether it may be initiated and maintained *in vitro*. Flower stem explants of *Tulipa* regenerated shoots only when taken from un-sprouted bulbs (Wright and Alderson, 1980). Adventitious shoot formation on cotyledons in conifers was affected by both the growth rate of the parent tree (Mott, *et al.,* 1977) and the size of the seed (Abo El-Nil, 1979). These factors will have been influenced by the previous years season and growth. A second issue is the taxonomy of the explant; dicotyledons and gymnosperms are highly variable and more recalcitrant more monocotyledons (Benson, 2000).

In vitro plants may also manifest life cycle responses of parent plants with environmental cues. Tropical plants from arid and semi-arid zones are physiologically adapted to wet and dry seasons and temperate species such as *P. sitchensis* have evolved complex seasonal responses to survive short day lengths, low light intensities and reduced temperatures (Benson, 2000). *P. sitchensis* has shown seasonal patterns in tissue culture (Selby and Harvey, 1985).

1.3.5.3 Micro level –epigenetic gene expression patterns and DNA methylation

Development processes in plants (such as during *in vitro* ageing, vernalisation or stress) via genetically controlled cues are regulated through changes in DNA methylation (the addition of a methyl group to deoxyribonucleosides, with deoxycytidine as the main methylation site). DNA methylation is a significant and widespread phenomenon: in some plant species up to 30% of total cytosine can be methylated. Gene expression is affected as DNA methylation alters the binding of transcriptional factors. Genetic expression has been correlated with a reduction in methylated cytosine, whilst inactivation was correlated with an increase in methylated cytosine (Klaas and Amasino, 1989). Reduced DNA methylation disrupts normal plant development. *Arabidopsis* with reduced DNA methylation levels displayed a number of abnormalities including reduced stature, altered leaf size and shape, reduced root length, reduced fertility and loss of apical dominance (Finnegan, *et al.,* 1995).

DNA methylation detection techniques

Global methylation and specific site studies show that variation in DNA methylation occurs frequently in the culture process (Kaeppler, *et al.,* 2000). Harding *et al.*, (1996), using restriction enzymes, determined that the methylation status of *Vitis vinifera* was altered as a result of *in vitro* manipulations. In carrot cultures, global methylation levels decreased with increasing kinetin but increased with increasing 2,4-D levels (Loschiavo, *et al.,* 1989). Changes in global DNA methylation levels support the hypothesis that developmental timing plays a role in affecting variation in methylation levels and patterns (Kaeppler, *et al.,* 2000). In this study global methylation studies will be used to determine whether growth instability in shoot-tip cultures is associated with changes in DNA methylation patterns. Protein content will also be determined, as methylation can affect protein synthesis.

1.3.5.4 The importance of culture environmental control

Technology transfer is a core factor in developing *in vitro* protocols, hence, control of physical factors such as culture vessel size and aeration, light, day length and temperature is critical to obviate recalcitrance. The control of these factors, involving technology transfer, is considered one of the most critical in international plant laboratory collaborations (Reed, *et al.,* 2004).

A number of studies (Hughes, 1981) have shown species/explant specific sensitivity to *in vitro* culture differences even at the microenvironment level (i.e. inside culture vessel). This specificity of optimisation is likely to be inter-related to the donor species/explant physiology as previously discussed. Critical factors are likely to interact to produce final phenotype differences but studies have been undertaken to consider the individual impacts affecting culture performance and these are discussed below.

(a) Temperature

Temperature controls both *in vivo* and *in vitro* plant growth and development and plants can only function and maintain normal metabolic activities within a certain temperature range which is species specific (Hughes, 1981). Temperature can influence the nutritional and hormone requirements of the *in vitro* plant and may alter morphogenic events. Saher, *et al.*, (2005) report that a culture vessel cooling system produced shoots that showed reduced hyperhydricity (characterised by a translucent, water-soaked appearance resulting in culture deterioration and lipid peroxidation) when compared to controls.

(b) Vessel ventilation

In vitro plant culture may be affected by the gas phase above the culture system and the relative humidity (RH). Cell systems are most affected, but not exclusively, by the gases ethylene, O_2 and CO_2. These gases may influence *in vitro* plant tissue growth, development and morphogenesis. The role of ethylene in *in vitro* culture is unclear but there are reports showing that high levels (50ppm) cause retarded growth and browning (Hughes, 1981). Ethylene will be discussed in section 1.3.7.5.

Increasing CO_2 and decreasing O_2 are reported to promote plant growth substantially. In potato varieties L.*Binge* (Buddendorf-Joosten and Woltering, 1996) and L.*Haig* (Cournac, *et al.,* 1992) *in vitro* plant growth was strongly promoted when the carbon dioxide concentration was increased from 0.05 % to 5%. The increases were also associated with increased leaf area and increased leaf numbers. Oxygen decrease from 21 to 3% produced a similar effect. Chlorophyll content in these plants was slightly less than controls. CO_2, O_2 and ethylene levels were always found to be similar (Hughes, 1981).

The relative humidity (RH) inside the culture vessels is generally very high (90-100%) as demonstrated by Buddendorf-Joosten and Woltering (1996). Lowering the RH (60%-70%) is not reported to influence plant growth substantially in potato (Buddendorf-Joosten and Woltering, 1996) or rose plantlets (Capellades, *et al.,* 1990).

The general effects of reduced ventilation have also been investigated. Micropropagated carnations in non-ventilated vessels showed thickened cuticles, decreased cell size and intracellular space size (Majada, *et al.,* 2000). Increased ventilation pushed the culture-induced phenotype closer to that of *ex vitro*-acclimatized plants. In *P. sitchensis*, inhibition of somatic embryo maturation was caused by butylated hydroxytoluene, a volatile released by parafilm (Selby, *et al.*, 1996b). Consequently all cultures maintained in this study were sealed using cling-film, which was determined not to produce potentially phytotoxic metabolites.

(c) Photoperiod

Plant tissue culture media support heterotrophic growth and there is not a requirement for light to support primary autotrophic metabolism *in vitro*. However, a light photoperiod at a specific intensity is still required in chloroplastic cell tissue to activate pigment receptors involved in hormone regulation. *In vitro* differences in light both in photoperiod and light intensity are likely to influence morphogenesis, development and shoot formation (Hughes, 1981). Although photoperiod can be tightly controlled, the optimal and robust regulation of light intensity becomes more difficult in different culture rooms. In *Begonia* x *hiemalis*

cultivars, shoot formation was increased as light intensity increased from 3000 to 6000 lux, but the dry weight remained the same indicating that the shoot formation was at the expense of shoot size (Welander, 1978).

1.3.5.5 Phenolics

Woody plant species with secondary thickening and lignification are associated with high levels of phenols. Sub-culture dissection and specific components in the medium (metal cations) may enhance phenolic oxidation, which is associated with *in vitro* recalcitrance. The result is browning or blackening of affected areas resulting in tissue necrosis and even explant death (Benson, 2000). If cell breakdown is involved vacuolar phenolics can freely mix with proteinaceous gene products (McCown, 2000).

1.3.6 *In vitro* plant ageing and loss of morphogenic capacity

Prolonged *in vitro* culture may lead to *in vitro* ageing. This can occur rapidly (over one subculture) or slowly (over years). In shoot cultures this is manifested by a decline in growth rate, senescence and hyperhydricity. The weakened resistance to stress and disease increases the probability of death. Free radical activity is associated with the hormonal control of senescence (Benson, 2000). Increased DNA methylation has also been associated with prolonged (1 year) culture of *Vitis vinifera* micropropagated shoot cultures (Harding, *et al.*, 1996). *In vitro* aged dedifferentiated cultures are associated with loss of morphogenic competency and development of neoplastic features at the cytological level (Hasler, *et al.*, 2003).

1.3.6.1 Juvenility and woody perennials.

A reduced *in vitro* response of mature tissues compared to juvenile tissue explants has been noted in several woody species (Benson, 2000). *In vitro* shoot proliferation, from adult material of *Myrtus communis* displayed hyperhydricity, whereas seedling-derived *in vitro* shoot proliferation showed none (Parra and Amo-Marco, 1998). In conifer species, somatic embryogenesis has only been possible from juvenile tissues, but *in vitro* maintenance is required over several years (7 yrs in *P. sitchensis*) during progeny testing. Conifer clonal foresters are faced with the problem of physiological maturation and can only proceed if tested material could be reverted to a juvenile form after testing or maintained in the juvenile form while testing occurred. A number of techniques have been tested and proposed (Samuel, *et al.*, 2000):

(1) Mature Sitka spruce scions were grafted on to juvenile rootstocks in an attempt to rejuvenate as has been achieved in various horticultural species (Hartmann and Kester, 2002). No rejuvenation was achieved even after 5 repeated re-grafts.

(2) Juvenile shoots were incubated at low (non–freezing) temperatures in an attempt to delay the maturation process, but the tissues degenerated rapidly.

(3) The effects of somaclonal variation, genetic instability and *in vitro* juvenility may be controlled by cryopreservation (storage in liquid nitrogen at -196°C).

When progeny have been tested the superior tested genotypes may be identified, withdrawn from cryostorage and propagated to supply plants for clonal forestry. Following two pilot projects on the cryogenic storage of Sitka spruce tissues, this study aims to produce a robust cryopreservation methodology applied to a broad range of genotypes.

1.3.6.2 Dedifferentiated cultures and embryogenic suspensor masses

Embryogenic cultures of many species including conifers require regular subculturing: liquid culture of *Picea glauca*, every week (Dunstan, *et al.*, 1993), solid culture of *Pinus sylvestris*, every two weeks, (Haggman, *et al.*, 1998) and of *Picea sitchensis*, every four weeks (John, *et al.*, 1995). There are many reports of embryogenic culture degradation and loss over time. Changes in embryogenic potential, growth, yield and morphology of subsequent somatic embryos of liquid embryogenic cultures of *P. glauca* have been reported within months of weekly subcultures (Dunstan, *et al.*, 1993). The retention of morphogenic potential of other woody species such as *Vitis* spp. during long-term maintenance has proved to be difficult (Wang, *et al.*, 2002). On solid medium, cultures lost embryogenic potential or differentiated completely and germinated, while on liquid medium, the medium browned and embryos yielded abnormal plantlets (Wang, *et al.*, 2002). A study of *P. abies* embryogenic cultures subcultured over several years showed phenotypic variation such as immature embryos with diffuse organisation, partly mature albino embryos and somatic seedlings comparable to dwarf mutants (Fourre, *et al.*, 1997). Genetic alterations such as somaclonal variation are often attributed to cell culture degradation (Scowcroft, 1984).

1.3.6.3 Tissue culture instability

Reports of tissue culture instability manifested at the karyotypic, morphological, biochemical and molecular levels have appeared since 1961 (Scowcroft, 1984).

(a) Chromosomal instability in tissue culture may be shown as variant cells with polyploidy and aneuploid changes, structural changes in chromosome morphology, mitotic aberrations including multipolar spindles, lagging chromosomes, fragments and asymmetric chromatid

separation (Bayliss, 1980). Bayliss (1980) examined 53 reports where chromosomal status of callus or suspension cultures were evaluated; only seven showed no variants. There is no clear cause of enhanced frequency of chromosomal abnormality in tissue culture but it is believed to be associated with disorganised growth. Partially differentiated cultures, such as those produced in conifer somatic embryogenesis, are reported to exhibit less variation than callus cultures (Wang, *et al.*, 1993). However in conifer embryogenic cultures: tetraploidy was detected in some *Pinus radiata* cultures using flow cytometry (Maddocks, *et al.*, 1995); in Norway spruce, altered ploidy was detected in 3 of 240 plants generated at 4 to 28 months of a five–year study of extended culture (Fourre, *et al.*, 1997).

(b) Morphological changes may occur amongst subclones of one parental line. In *Citrus grandis* callus grown under identical culture conditions one subclone formed embryoids and the other formed shoots (Chaturverdi and Mitra, 1975). Other varying parameters in callus growth included growth rate differences, friability, sliminess and pigmentation (Scowcroft, 1984).

(c) Biochemical changes determined through secondary metabolite production and isoenzyme analysis have been used to detect changes in tissue culture (Scowcroft, 1984). Subline differences between nicotine production in *Nicotiana rustica* and *N.tabacum* cultures were reported (Tabata, *et al.*, 1978). In *P. engelmanni* complex, no evidence of isozyme variation between sublines was detected (Eastman, *et al.*, 1991).

(d) Molecular level changes may be monitored through three main methods (Harding, 1999): (1) polymerase chain reaction (PCR) based methods such as Random Amplified Polymorphic DNA (RAPDs); (2) DNA-DNA hybridization methods such as Restriction Fragment Length Polymorphisms (RFLPs) and Amplified Fragment Length Polymorphisms (AFLPs); (3) DNA methylation studies. No variation in RAPD profiles was determined between three *P. mariana* lines (Isabel, *et al.*, 1993).

Any tissue culture instability manifestations and their detection methods may be determined following cryopreservation and it is desirable that the impact of cryopreservation stability can be assessed. Nuclear and chloroplast DNA in plants regenerated from cryopreserved shoot-tip cultures of potato was comparable with control cultures when DNA-DNA hybridization methods were applied (Harding and Benson, 2000). RFLP fingerprint analysis applied to embryogenic culture of interior spruce before and after cryopreservation showed no variation (Cyr, *et al.*, 1994).

When these instabilities are manifested as heritable mutations amongst the progeny of regenerated plants they are termed somaclonal variation. Somaclonal variability is affected by many factors including the developmental state, ploidy level of the explant source, physical handling of cultures, duration of culture, frequency and number of subcultures (Cyr, 2000). There are a number of factors that may cause somaclonal variation including DNA methylation and free radical damage generated through oxidative stress.

Attempts to evaluate the level of somaclonal variation in conifer embryogenic cultures have shown mixed results. No evidence of variation was detected in sublines at isoenzyme level or amongst genotypes after cryopreservation using RFLP in interior spruce (Cyr, *et al.*, 2000). However, in two lines of hybrid larch, 70% of cells showed hyperploid number of chromosomes and cultures with a high level of aneuploidy did not regenerate mature somatic embryos. Altered ploidy was observed in *P. abies* in 3 of 240 of plants generated at 4 to 28 months of a five–year study (Fourre, *et al.*, 1997). *In vitro* embryogenic clones of *Picea glauca* showed somaclonal variation (using randomly amplified polymorphic DNA fingerprints) at 2 and 12 months in culture, but no variation was observed in corresponding regenerated trees (DeVerno, *et al.*, 1999).

1.3.6.4 Loss of regenerative capacity and morphogenic potential

Besides general culture deterioration over time in culture, cell and tissue cultures may also lose their ability to undergo total regeneration. The decline can be over one subculture or over several years, depending on the species and maintenance conditions. In spring and winter rye, the morphogenic potential of embryogenic callus was lost after eight months (Ma and Pulli, 2004). In *P. mariana* and *Larix* cultures, only a 10% embryogenic recovery was observed following 14 to 22 weeks of liquid culture (Charest and Klimaszewska, 1995). Little work has been undertaken to determine how long morphogenic competency can be maintained in embryogenic cultures of *P. sitchensis,* maintained on solid medium, and this is an issue that will be addressed in this study. Three theories have been proposed to explain declines in regenerative capacity and morphogenic competence (George, 1993):

(1) Morphogenesis results only from competent meristematic cells that are descendants of the source explant population (Warren, 1991). If these cells cannot compete with morphogenic cell types that are better adapted to culture growth, they will be diluted out. Morphogenic ability may be lost in these callus cells through aneuploidy and polyploidy (Gasper *et al.*, 2000). Other karyotypic alterations may have even more impact on the decline of totipotency and regenerative capacity.

(2) Morphogenic competence may be influenced by the reduction or accumulation of organogenesis promoting compounds that were present in initial explant derived cultures. There are reports of increased auxins in cultures over time resulting in the promotion of de-differentiation (Warren, 1991).

(3) There may be an epigenetic change in cultured cells: a change in gene expression patterns brought about by the culture environment. Genes may be hyper– or hypo– methylated with resultant expression modification or there may be a change in endogenous hormone metabolism, receptivity or transduction pathways.

Neoplastic progession explains the loss of regeneration capacity of too long subcultured hormone-dependant calli (Hasler, *et al.,* 2003). At the end of neoplastic progression plant cells have probably undergone several independent genetic accidents with cumulative effects (Gasper *et al.*, 2000), characterised by oxidative stress–induced, atypical biochemical pathways and may be considered fully habituated.

1.3.6.5 Habituation– neoplastic progression and plant cancer

Habituated cultures are those that are able to grow without plant hormones and are able to continuously divide (Hagege, 1996). The phenomenon can be gradual or arise spontaneously and may generate a neoplastic state (Gaspar, 1995). Habituation is a widespread problem affecting many plant species within agriculture and horticulture. Plant cancer has been defined as the irreversible loss of organogenic totipotency (Gaspar, 1995). Although there are many possible explanations for autonomous cell division, including the continuous production of auxins and cytokinins, altered ethylene metabolism or gene expression, and free radical interactions, the mechanism of habituation is still unclear.

1.3.7 Oxidative stress and associated biochemistry

Oxidative stress is implicated in plant *in vitro* stress; degeneration of cultures, senescence, developmental stress and ageing are especially likely to influence dedifferentiated woody species cultures that have been maintained for a long time (2+ years) such as the Sitka spruce ESM in this study.

Oxidative stress is encountered by all aerobic organisms mediated by reactive oxygen species, and aerobes have consequently developed complex antioxidant systems to combat damaging

free radicals and their toxic reaction products. Plants are susceptible to oxidative stress for two main reasons (Benson and Bremner, 2004): photosynthesis drives water splitting reactions where O_2 is the terminal electron acceptor in plant respiration, and O_2 and reactive oxygen species participate in pathogen defence via plant secondary metabolism. Reactive oxygen species play an essential role in plants but oxidative pathways can be disrupted under stressful conditions. Photosynthetic organisms, such as woody plants, maintained under 'artificial' *in vitro* heterotrophic conditions are especially at risk.

A radical is any species containing one or more unpaired electron. Free radicals can be formed in a number of ways: addition of an electronloss of an electron or cleavage of a bond. Radical and non-radical species of oxygen can be derived from ground state oxygen ($^3O_2$) by the addition of energy or electrons.

Ultraviolet (UV) energy can convert $^3O_2$ into the more reactive singlet oxygen $^1O_2$. Addition of one electron to $^1O_2$ leads to the formation of the superoxide radical ($O_2\cdot^-$). When two electrons and two protons are added to $O_2\cdot^-$ hydrogen peroxide (H_2O_2) is produced.
The hydroxyl radical (·OH) is considered the most reactive of the oxygen species, and is generated from a series of reactions. The most common source of hydroxyl radicals biologically is via the Fenton reaction as shown in Figure 1.5 a. Plant systems produce hydrogen peroxide via a number of pathways and in addition to a form of ferrous ion hydroxyl radicals may be readily generated (Fig 1.5 b). Enzymes may produce hydrogen peroxide directly. The most common group of enzymes to do this are the superoxide dismutase (SOD) group.

Figure 1.5a Fenton reaction pathway of hydroxyl radical production (Benson and Bremner, 2004)

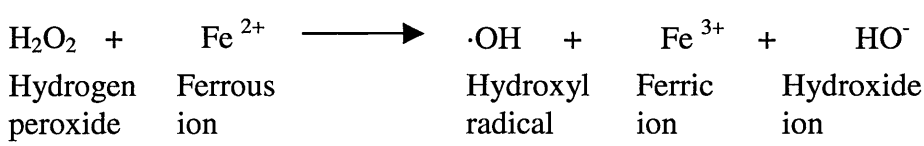
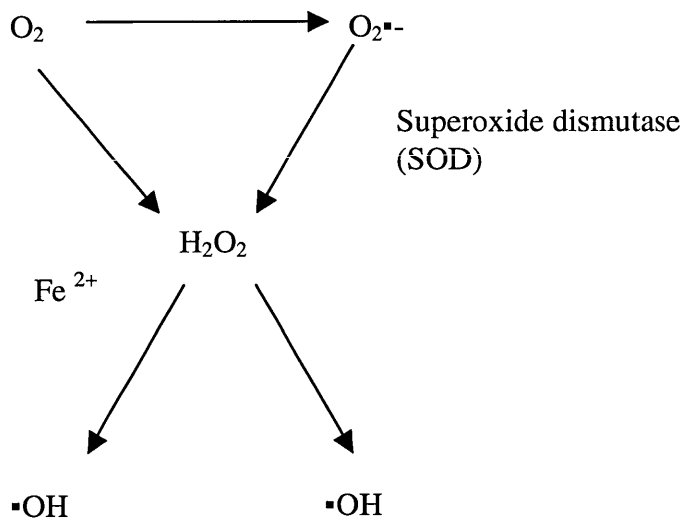


Figure 1.5b Reactive oxygen species generation from O_2 (Benson and Bremner, 2004)



1.3.7.1 Oxidative stress in the *in vitro* environment

A number of researchers have proposed that oxidative stress may be involved with *in vitro* responses that may have particular importance to woody plants (Adams, *et al.*, 1999, Benson and Roubelakis-Angelakis, 1992). Oxidative stress is associated with many factors of *in vitro* plant growth including; heterotrophic conditions, artificial light, and reduced ventilation. *In vitro* manipulations, such as routine sub-culturing, have been specifically associated with the production of active oxygen species.

Antioxidant investigations have been previously applied to *in vitro* woody plant callus culture investigations. Benson and Roubelakis-Angelakis (1992) determined that a reduction in fluorescent oxidation products in the latter stages of callus induction was concomitant with increased antioxidant activity. In *Pinus sylvestris* callus cultures, a decline in growth rate, browning, a degradation of cell membranes and cellular disorganisation between the 2nd and 3rd weeks of culture was associated with an increase in peroxidase activity (indicating lipid peroxidation) (Laukkanen, *et al.*, 2000). *Pinus virginiana* 'browning' callus cultures, showing decreased plant regeneration via organogenesis, were associated with an increase in lipid peroxidation and polyphenol oxidase and a decrease in antioxidant enzymes; ascorbate peroxidase, glutathione reductase and superoxide dismutase activities (Tang and Newton, 2004). No investigations of oxidative stress in *in vitro P. sitchensis* cultures have been reported.

1.3.7.2 Lipid peroxidation

The processes of lipid peroxidation can produce some of the most damaging reactive species and products that will cause cellular and cytological damage. The process of lipid peroxidation is caused by hydroxyl radicals (·OH) reacting with lipid membranes (Benson and Bremner, 2004). Activated radical CH_2 groups of polyunsaturated fatty acids are attacked to generate lipid free radicals which react with oxygen to form lipid hydroperoxides (LOOHs). LOOHs may be reduced to hydroxyl acids such as hydroxyoctadecadienoic acids (HODEs), under biological conditions, and used as lipid peroxidation markers. LOOHs may also decompose into toxic and mutagenic secondary reaction products such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). MDA may be responsible for mutagenic lesions following DNA base interactions. HNE is genotoxic and chemotoxic, and is reported to inhibit callus growth (Muller, *et al.*, 1996).

Other compounds such as ethane and ethylene are also produced. The hydroxyl radical (·OH) readily reacts with dimethyl sulphoxide (DMSO) to produce the methyl radical and methane. Methane, ethane and ethylene can be detected using gas chromatography (GC) and therefore are indirect indicators of oxidative stress (Benson and Bremner, 2004).

1.3.7.3 Proteins and free radicals

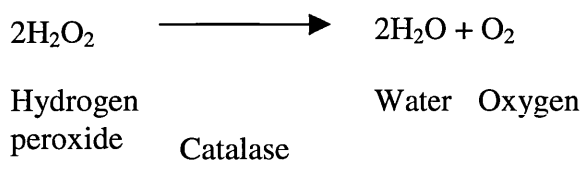
MDA can react with proteins containing lysine residues and cross-links formed between protein chains. HNE, an unsaturated hydroxyaldehyde may form adducts with DNA, proteins, phospholipids and amines. These reactions may alter the structure and function of proteins and enzymes causing a profound effect on cell stability and metabolism (Muller *et al.*, 1996).

1.3.7.4 Antioxidants

Plant antioxidant defence mechanisms comprise superoxide dismutases (SODs); catalase and 'guaiacol'-peridases, ά –tocopherol (vitamin E), ascorbate peroxidases, mono or dehydroascorbate reductases, glutathione peroxidases, and glutathione reductases (Elstner, *et al.*, 1993). Several examples of the mechanisms of antioxidant protection are outlined.

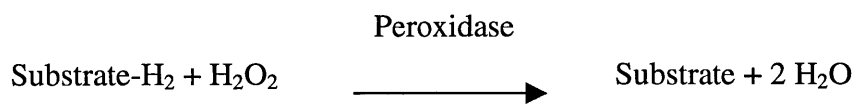
In example 1, catalase (Fig. 1.6a), a tetrameric iron porphyrin (haem) protein is used to remove the cytotoxic H_2O_2. SODs types are classified according to their sub-cellular location and metal co-factor. In example 2, SOD antioxidants convert the superoxide radical anion into hydrogen peroxide and oxygen (Fig. 1.6b).

Figure 1.6a Action of catalase on hydrogen peroxide (Benson and Bremner, 2004)



Antioxidants derived of haem-containing proteins that catalyze the H_2O_2-dependent oxidation of substrates including phenolics (e.g. guaiacol) are called peroxidases.

Figure 1.6b Action of peroxidase



H_2O_2 scavengers are primarily found in the chloroplast.

Vitamin E (ά-tocopherol) is lipophilic and is found embedded in membranes in close proximity to potential lipid peroxidation sites, and is preferentially oxidized compared to polyunsaturated fatty acids. Ascorbate cycling mechanisms regenerate ά-tocopherol. Glutathione protects oxygen sensitive enzymes from the oxidative degradation of the sulfhydryl groups.

1.3.7.5 Ethylene

Ethylene is produced as an endogenous plant hormone and can be evolved exogenously from tissue culture medium, especially when the other plant hormones auxins and cytokinins are included. Ethylene is also a decomposition product of lipid peroxidation, as hydroxyl radicals attack any activated CH_2 groups of polyunsaturated fatty acids generating lipid free radicals and eventually lipid hydroperoxide (Benson and Withers, 1987).

Ethylene, generated by *in vitro* plant growth tissues, may influence growth and development as it accumulates in culture vessels, especially in rapidly growing non-differentiated or partially-differentiated cultures (Biddington, 1992). Ethylene generally appears to inhibit shoot regeneration and somatic embryogenesis. In *Picea abies*, ethylene production was higher in non-embryogenic callus cultures than in embryogenic callus, and the authors inferred that a high rate of ethylene production may be associated with a lack of morphogenic potential in conifer cell cultures (Wann, *et al.*, 1987). Non-embryogenic suspension cultures

of *P. glauca* also accumulated higher levels of ethylene than embryogenic cultures and formation of pro–embryos was inhibited by incubating the cultures with high ethylene levels (Kumar, *et al.,* 1989).

Ethylene can bc analysed quantifiably by volatile headspace sampling and GC (Benson and Withers, 1987). Careful interpretation of ethylene levels is required as ethylene biosynthesis and action requires intact membranes; a lower level of ethylene production may also indicate membrane damage, while an increase may indicate membrane recovery.

1.3.7.6 Oxidative stress and associated chemistry detection methods

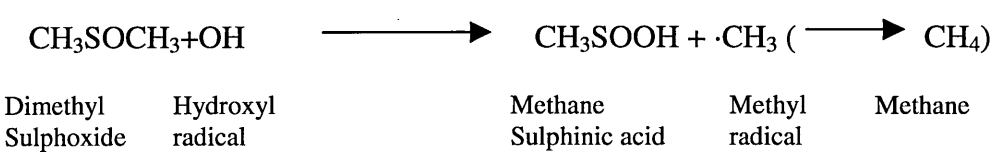
A number of direct and indirect plant oxidative stress detection methods have been developed and are suitable for *in vitro* monitoring.

(1) Free radical formation detection

DMSO

DMSO may be used as a hydroxyl radical probe (Benson and Withers, 1987), as shown in Fig. 1.7.

Figure 1.7 DMSO as a hydroxyl radical probe



Antioxidant assays provide accurate indirect methods to measure oxidative stress status. The total aqueous antioxidant activity assay measures ascorbate, glutathione, SH groups associated with proteins and amino acids, polyamines, phenolics and alkaloids and is an efficient preliminary antioxidant determination technique that will be used in this investigation.

(2) Secondary oxidative stress detection–lipid peroxidation products

Oxidised fatty acid reaction products, from free radical attack of lipid membranes, can be used as oxidative stress markers (Benson, 1990). Reaction products include volatile hydrocarbons (such as ethylene), conjugated dienes, lipid peroxides and aldehyde breakdown products.

Ethylene

Ethylene can be analysed by volatile headspace sampling and gas chromatography along with other small-chained carbon products of lipid peroxidation such as methane, ethane and pentane (Benson, *et al.*, 2006 In press). Ethylene production in *in vitro* culture volatile headspace will be investigated using gas chromatography methodology in this study.

Schiffs bases

Other aldehydic reaction products of lipid peroxides can cross-link with protein groups to form Schiffs bases that may be measured through assay techniques

Protein content

Protein content may be an indication of growth/vigour of *in vitro* cultures and may indicate oxidative stress as interference in gene translation products as well as the overall gene expression (Harding, 1994). There are several methods of protein detection for plant tissue including UV absorbance and chromatographic methods (Schulten and Schnitzer, 1998), quantification of α-NH_2-N by the ninhydrin method, (Stevenson and Cheng, 1970), the micro-Kjeldahl procedure (Young, 1963) and calorimetric protein assay techniques such for total soluble protein content determination in this study, the Coomassie method (Bradford, 1976).

1.4 Cryopreservation

Cryopreservation is the preservation of viable cells, tissues and organs in liquid nitrogen, at -196°C (Benson, 1999a) or at vapour phase nitrogen temperatures (-135°C to -150°C) (Stacey, 2004). To utilise cryopreservation researchers first discovered the mechanisms of freezing and cryoprotection. The principles of cryobiology and damage to biological systems from ice nucleation and formation were clarified by Mazur's two-factor hypothesis which proposed that cell injury was the result of either the concentration of solutes by extracellular ice, producing 'solution effects', or resulted from the presence of intracellular ice, termed mechanical injury (Mazur, 1965, 2004).

1.4.1 Ice formation

Water can freeze at 0°C in the presence of an ice nucleating agent, but without such an agent it may be super-cooled to -40°C. At this point water molecules form clusters and it is defined as the 'temperature of homogenous ice nucleation' (Meryman and Williams, 1984). When freezing does occur it produces an exothermal event termed 'the latent heat of freezing.' The

latent heat may trigger ice nucleation in super-cooled water. As ice is fused the temperature in the liquid will rise to 0°C and remain until all the water is frozen out in the case of pure water. In a solution the freezing point is depressed and continues to be depressed as the pool of freezable water becomes less and the solutes are concentrated.

1.4.2 Freezing injury

In early biological studies (1920-30s) freezing injury was believed to be predominantly because of the direct damage by ice crystal formation (Luyet and Gehenio, 1940). Lovelock, (1953) was the first researcher to focus on the clear correlation between the concentration of extracellular solutes and cell death. The current understanding of freezing injury in both plant and animal cells is based on Mazur's two-factor hypothesis (Mazur, 1965) proposing that cell injury was the result of, 'solution effects,' or from the mechanical injury caused by intracellular ice. Mazur also proposed that the major factor in determining whether or not cells survived freezing to a low temperature is the rate at which they are cooled (Mazur, *et al.*, 1970).

Mechanical injury

Ice usually forms extracellularly first in natural plant systems. Slow cooling generally produces extracellular ice and may cause cell rupture as demonstrated in naked protoplasts, (Steponkus, 1982). Intact cell membranes are generally thought to be impenetrable to ice, and the primary mechanical damage is through intracellular ice formation. At fast cooling rates, large intracellular ice crystals cause mechanical damages.

Solution effects – cell injury from osmotic dehydration and volume change

Damage arising from ice crystal formation may also occur through dehydration. At a slow cooling rate, where extracellular ice first appears, intracellular unfrozen water is drawn from the inside to the outside of the cell to compensate for the water vapour deficit. The damaging effects of osmotic stress are volumetric/area contraction, a concentration of intra-extra-cellular compounds, eutectic crystallisation and changes in pH and enzyme activities (Steponkus, 1984).

Inverted U

The inverted U hypothesis was named as such because plots of survival vs. cooling commonly take the form of an inverted U (Mazur, 2004). Maximum survival occurs at an intermediate rate. If the cooling rate is too high or too low then fewer cells survive. A range of cell types have been tested that correspond with this hypothesis including; yeast (Lepock,

et al., 1984), mammalian sperm (Mazur and Koshimoto, 2002) and mouse marrow stem cells (Leibo, *et al.,* 1970). At optimal rates cryoprotectants, in proportion to their concentration, can be used to protect cells.

1.4.3 Ice and glass formation in biological systems: principles for cryopreservation

In biological systems, there are three responses to freezing according to the rate of freezing, intracellular solute concentration and viscosity:
(1) Progressive freezing initiates ice-nucleation extra-cellularly. In plant cells, ice penetrates the cell wall but not the plasmalemma (Kartha, 1987). A water deficit is created between the inside and the outside of the cell causing remaining intra cellular water to migrate to the extra cellular environment. This causes cell dehydration, a reduction in cell volume and an increase in intracellular solute concentration. The faster the cooling, the greater the number of ice nucleation events.

'Slow freezing' dynamics form the basis of traditional cryoprotection and controlled rate cooling (Meryman and Williams, 1984). Through precise control of the freezing rate, plant cells may be dehydrated sufficiently to prevent cell death when they are immersed in liquid nitrogen. Cryoprotectant application is also required to stabilize cell components through colligative action. Colligative cryoprotectants, such as glycerol and dimethyl sulphoxide (DMSO), penetrate the cell and are distributed throughout intra- and extra-cellular domains. This prevents toxic solute accumulation in cell compartments. Optimised colligative cryoprotection depresses the freezing point so that insufficient water is present in the cell system to cause fatal damage. Prior to cooling, traditional cryopreservation protocols often include a dehydrating or cell hardening pre-treatment to reduce the freezable water (but not to a critical level) and facilitate controlled rate cooling.

(2) Rapid freezing, as in direct LN immersion, nucleates inter- and intra-cellular ice simultaneously; this is often fatal for the cell.

(3) Vitrification is the phase transition of liquid to an amorphous 'glassy state', (Benson, 1999a). In the case of water, a remarkably stable solid without a crystalline structure is produced. A vitrified solution is not subject to the evaporative effects of osmotic concentration, due to extracellular ice formation in equilibrium freezing. Intracellular ice formation is precluded in a vitreous solution. Vitrification is an important route for plant germplasm cryoprotection and there are a number of methods by which cells can be induced to undergo vitrification:

(a) *Desiccation based*

Plant tissue may be desiccated to a critical moisture content, where sufficient viscosity is formed to circumvent ice formation and promote glass formation. Desiccation may be achieved by sterile air-flow or by drying over silica gel (Sherlock, *et al.,* 2005) or salt solutions for an optimised duration. This method is suitable for desiccation tolerant species/explants only and survival may be improved by pre-encapsulating the explant in a calcium alginate matrix (Fabre and Dereuddre, 1990). A number of protocols rely on an osmotic agent pre-treatment, such as sucrose, before desiccation treatment (Dumet, *et al.*, 1993).

(b) *Chemical based*

The second route for vitrification-based cryopreservation involves the application of highly concentrated non-penetrating and penetrating cryoprotective additives and cryoprotectants to promote a balance of osmotic cell dehydration and cell stabilisation. Plant Vitrification Solution Number 2 (PVS2) was developed by Sakai, *et al.*, (1990) and is composed of ethylene glycol, DMSO and glycerol. At the high molarities required for cryoprotection it may be toxic to some species/explants. Therefore critical optimisation factors of the PVS2 method include the application time and temperature of application. Another critical factor may be the post-thaw removal or 'unloading' of the toxic solution, which must be undertaken without causing osmotic shock.

(4) *Rewarming – on ice re-crystallisation*

In slowly cooled cells, ice re-crystallisation on rewarming mirrors the ice nucleation events of cooling (Mazur, 2004). However in rapidly cooled cells, if re-warming is slow, small ice crystals formed during cooling may enlarge to a damaging size. In vitrified glasses, if re-warming is too slow the glass may de-vitrify and lethally freeze the cells. The standard re-warming technique is to immerse cooled vitrified samples into a 45°C water bath, but in some cases a two-stage re-warming is recommended to circumvent damaging glass relaxation events. A slow warming step, to a temperature below glass transition followed by a rapid warming step to stop de-vitrification is recommended (Benson, 1999a). Glasses obtained using encapsulation-dehydration may be more stable, and better able to cope with ambient re-warming, than PVS2 solutions.

1.4.4 Cryopreservation protocol components

1.4.4.1 Pre-treatments/culture

Many of the protocols designed for controlled rate cooling and vitrification-based cryopreservation require a pre-treatment. Before the details of cryopreservation applications are discussed, the principles and empirical application of pre-culture processes (cold hardening), osmotica (sucrose, sorbitol), plant growth regulators (ABA) cryoprotective additives (polyethylene glycol) and cryoprotectants (DMSO) will be reviewed.

Withers (1985) evaluated the pre-growth of cell cultures in the context of cryopreservation tolerance. The time frame in the growth cycle at which cells are harvested can influence cryotolerance. Four stages were defined: (1) a lag phase cellular structure changes such as cytoplasmic component synthesis and vacuolar volume decrease, (2) exponential period of growth, until nutrients depleted, (3) a decline in cell division, and (4) a stationary phase at which time cells undergo increases in vacuolar and cellular volume. Freeze tolerance has widely been reported to occur towards the end of the lag phase and in early exponential growth. Peak TTC viability and corresponding peak respiratory performance of control cultures occurred after a peak in freezing tolerance. It was also determined that *Acer pseudoplatanus* suspension cells that had recently passed through mitosis (perhaps in G_0 phase) were more tolerant of freezing than similar sized or smaller sized cells at other phases. The time frame of pre-culture and cryopreservation is therefore an important parameter.

(a) 'Natural' mechanisms – cold hardening and dormancy

Freezing tolerance may be induced by low, but non-lethal temperatures above freezing. During the growing season many woody perennials cannot tolerate temperatures of -3°C but when they are fully hardened in the winter they tolerate temperatures as low as -196 °C (Chang and Reed, 2000). Cold acclimation is associated with many physiological and biochemical alterations, including membrane and protein composition changes, increases in sugar content, changes in plant hormone concentrations and alterations in gene expression (Crowe, *et al.*, 1990).

Sakai (1983) showed that the most cold-hardy coniferous genera such as *Picea*, *Tsuga*, *Pseudotsuga*, *Cedrus*, *Keteleeria* and *Pinus* utilise supercooling to ensure winter survival of buds. Hardy twigs and shoot primordia are thought to utilise extra-organ freezing, allowing freezing in cells surrounding the meristematic dome and the gradual removal of water to a vitreous state. This may explain why hardy conifers survive winter temperatures of -60°C and below. Strongly associated with these events is the synthesis of raffinose family

oligosaccharides, involving and alpha-galactosyl linkage to sucrose, via myo-inositol, to form the trisaccharide raffinose. It is thought that raffinose family oligosaccharides play some special cryoprotective role during periods of freezing temperatures (Cox and Stushnoff, 2001).

Cold treatments (cold hardening or cold acclimation) may be incorporated into the pre-culture phase of cryopreservation protocols (Table 1.1). Metabolic changes caused by desiccation and low temperatures are similar to those produced by sucrose and other disaccharide pre-treatments, and may result in similar mechanisms, such as membrane stabilization (Chang and Reed, 2000). Low temperature pre-treatments have been shown to improve the recovery of cryopreserved *in vitro* grown shoot tips. Conditions used for cold acclimation include constant low temperature with short photoperiods, total darkness or alternating temperatures with short photoperiods. Optimisation is required to manipulate the physiological conditions in target plant species whereby plants may be successfully cryopreserved but can also be triggered to re-develop following treatment.

In vitro shoot tips of *Pyrus* sp. were successfully cryopreserved using either 16wks of an alternating temperature (22°C + 8hr or 16hr light/-1°C darkness) or a constant temperature (4°C with an 8hr photoperiod or darkness) prior to controlled rate cooling (Chang and Reed, 2000). Cryopreservation of conifer shoot material has been achieved in the dormant buds of *P. sylvestris;* the best viability was obtained in buds collected in January or April (Kuoksa and Hohtola, 1991). Conifer *in vitro* shoot cold acclimation and *in vitro* dormancy studies have not yet been reported.

Table 1.1 Summary of cryopreservation methods applied to woody species

Woody plants	Explant	Method of cryopreservation	Pre-culture & Cryoprotectant	Response	Reference
Malus domestica	1) Dormant buds 2) *In vitro* shoot tip apices	1) Two –step freezing 2) Vitrification 3) Encapsulation-dehydration	1) PVS3 + controlled rate freezing (-0.1°C) 2) PVS3 3) 0.1-0.7M suc+ 1.0M bead pre-culture+4hr LA	Bud break or shoot –tip recovery	Wu, *et al.*, (2001)
Pyrus L.	*In vitro* shoot tip apices	Encapsulation-dehydration	Alternating –temperature cold-acclimation	Shoot-tip recovery and re-growth	Chang and Reed, (2000)
Pinus sylvestris	Partly dormant buds	CRC	1°C/min cooling until -40°C, LN for 10 min and -80°C storage	FDA viability	Kuoksa and Hohtola, (1991)
Betula pendula	*In vitro* shoot tip apices	CRC	Alternating –temperature cold-acclimation + 10^{-4}M ABA	Shoot re-growth	Ryynanen, (1998)
Vitis vinifera	*In vitro* shoot axillary buds	CRC or two step-freezing	3 to 4mths no subculture + 1mth 5°C	Shoot re-growth	Zhao, *et al.*, (2001)
Vitis. Spp.	Embryogenic cell suspensions	Encapsulation-vitrification	0.25-0.75M sucrose preculture Encapsulation 1hr PVS2	FW increase comparable to control by 2nd sub	Wang, *et al.*, (2004)
Mangifera indica L.	ESM	(1) Encapsulation-dehydration (2) Pre-growth-dehydration (3) PVS3	24hr 0.5M sucrose	(1) None (2) 8% recovery (3) 95% recovery	Wu, *et al.*, (2003)
Olea europea	SE	(1) Encapsulation-dehydration (2) Encapsulation-vitrification	4 days pre-culture 0.75-1.25M sucrose (1) 4hr desic. (2) 3hr PVS2	Plantlets and 2nd ESM	Shibli and Al-Juboory, (2000)

PVS3= Plant Vitrification Solution 3= Liquid culture medium + 50% glycerol + 50% sucrose (w/v). ABA= Abscisic acid; PVS3= Plant Vitrification Solution 3

(b) Pre-culture osmotica

Supplementation of the culture medium with osmotically active compounds may assist cryopreservation by inhibiting cell expansion, reducing cell and vacuole size, reducing cell water content and inducing freeze tolerance physiological and morphological aspects. Withers and King, (1977) first used 1.1 and 3.3% (w/v) mannitol as a pre-culture additive. This addition improved survival potential, extended the viable cell growth period and reduced mean cell size in *Acer pseudoplatanus*. Pritchard in 1982, also investigating *A. pseudoplatanus* culture cell size, determined the proportion of cells with a diameter of 25μm or less rose from 55%, in controls, to 79% in those treated with mannitol (Withers, 1985). In these maniitol treated cells, vacuolar volume was reduced from 34 to 14% of the total cell volume. Interestingly a single large vacuole in control cells was replace by several smaller vesicles in mannitol pre-grown cells. These cells showed depressed respiration and growth (dry weight measurements) and cell walls were also observed to be thinner cells pre-treated with 6 % (w/v) mannitol.

Picea species embryogenic suspension culture and somatic embryo pre-culture protocols are shown in Table 1.2. *P. sitchensis* ESM cultures have been cryopreserved using 0.4M sorbitol for 48hr as a pre-culture (Find, *et al.*, 1993). To date, no successful conifer *in vitro* shoot-tip culture cryopreservation protocol has been achieved. A range of pre-culture techniques have been applied to cryopreserve woody species explants (Table 1.1) including *in vitro* shoot-tip cultures of *Malus* domestica using 0.1-0.7M sucrose (Wu, *et al.*, 2001).

Sucrose, trehalose, sorbitol and proline

Various concentrations of sucrose and sorbitol have been selected for pre-culture in *Picea* cryopreservation (Table 1.2) Proline is naturally produced by plants under stress and is not reported to be toxic at pre-culture concentrations. In this section at these concentrations, pre-culture compounds are not considered as cryoprotectants.

Table 1.2- Summary of cryopreservation methods applied to *Picea* spp.

***Picea* species**	**Explant**	**Method of cryopreservation**	**Pre-culture & Cryoprotectants**	**Recovered Explants**	**Reference**
P. abies	ESM	CRC	PEG, Glu, DMSO	SE, PL	Gupta, *et al.*, (1987)
P. abies	ESM	CRC	Suc., DMSO	SE, PL	Galerne and Dereuddre, (1988)
P. glauca	ESM	CRC	Sorb., DMSO	SE, PL	Kartha, *et al.*, (1988)
P. mariana	ESM	CRC	Sorb., DMSO	SE, PL	Klimaszewska, *et al.*, (1992)
P. sitchensis	ESM	CRC	0.4M Sorb,. 10% DMSO	SE.,PL	Find, *et al.*, (1993)
P. mariana	ESM	Vitrification	0.8M sorb + 48hr PVS2	SE	Touchell, *et al.*, (2002)
P. glauca and *P. glauca* x engelmanni)	SE	Desiccation + LN immersion	Naked embryos desiccated over known salt solutions	PL	Percy, *et al.*, (2001)
P. mariana and *P. glauca*	SE	Desiccation + LN immersion	Naked embryos desiccated at 88–97% humidity	2^{nd} ESM + PL	Bomal and Tremblay, (2000)

ESM = Embryogenic suspensor masses; CRC= Controlled Rate Cooling; PEG= Polyethylene Glycol

Glu= Glucose; SE= Somatic embryos; PL= Plantlets; LN =Liquid Nitrogen

The mode of action of sugars and sugar polyalcohols in cryoprotective media is thought to be mostly dehydrative. They are considered to be extracellular cryoprotectants and are thought not to enter the cell. Kinetically, extracellular sugars may be useful, as a result of rapidly increasing viscosity with increasing concentration and decreasing temperature thereby reducing the cooling rate throughout the sample. Sivakumar, *et al.*, (2002) demonstrated that another important role of sugars and sugar alcohols in plants exposed to salt stress was to curtail oxygenase activity of the plant enzyme Rubisco. This may prevent wasteful photorespiration resulting in the overall reduction of plant productivity.

Studies of the physical chemistry of sugar and sugar polyalcohols at supercooled temperatures have been undertaken. Turner, *et al.*, (2001a) proposed that the action of polyalcohols was related to the OH groups arranged along one side of the membrane bilayer allowing more efficient binding and consequently providing better survival during cryopreservation. Table 1.3 shows the various properties of pre-culture additives. Crowe, *et al.*, (1990) reported that the efficiency for cryopreservation varies from one sugar to the next. By testing the structural preservation and functional integrity of membranes at low water activities the most effective sugar was found to be trehalose. Wang and Haymet (1998) determined, using DSC, that trehalose also showed the highest glass transition temperature among the four sugars.

Table 1.3 Characteristics of pre-culture sugars, polyalcohols and amino acids

Name	**Type**	**MW (g)**	**Formula**	**Total OH number per molecule**
Sucrose	Disaccharide	342.3	$C_{12}H_{22}O_{11}$	8
Trehalose	Disaccharide	342.3	$C_{12}H_{22}O_{11}$	8
Sorbitol	Polyalcohol	182.2	$C_6H_{14}O_6$	6
Proline	Amino acid	97.12	$-N-(CH_2)_3-CH-$	Not determined

Trehalose has been demonstrated to stabilise cell membranes, particularly the phospholipids bilayer and membrane proteins during cellular dehydration and freezing (Turner, *et al.*, 2001a). Trehalose occurs in a large variety of organisms, ranging from bacteria to invertebrate animals, where it serves as an energy source or stress protectant (Wingler, 2002). Its synthesis has been most noticeable in desiccation-tolerant 'resurrection' plants. The mechanism by which trehalose protects cells during freezing and desiccation stress is not fully understood. The exogenous addition of trehalose to liquid cultures of *Arabidopsis* seedlings rapidly altered disaccharide levels and induced detoxification and stress response proteins (Bae, *et al.*,

2005). Bae suggests that trehalose functioned as an elicitor of genes involved in biotic and abiotic stress.

Trehalose appears to preserve biological membranes in the absence of water (Crowe, *et al.*, 1985). When vesicles of calcium-transporting membranes (sarcoplasmic reticulum of lobsters) were dehydrated with trehalose there was no evidence of morphological damage. When membranes were re-hydrated they were able to transport calcium normally. In the absence of trehalose the same vesicles showed that the phospholipids formed complex crystalline phases during dehydration and were unable to function normally upon rehydration. Trehalose is thought to stabilise dry membranes by hydrogen-bonding with polar head groups of membrane phospholipids and hence replacing the water around the polar residues (Crowe, *et al.*, 1985).

Spectroscopic evidence on the interaction of trehalose and dipal-mitoyl-phosphatidylcholine indicated that trehalose may hydrogen bond to the hydration shell in the phospholipid head group (Rudolph and Crowe, 1985). DSC and monolayer studies showed that trehalose inhibited the induction of gel phases by reducing water content, and the spread of the phospholipid monolayers.

Proline has been used as a pre-growth additive and as a cryoprotectant (Withers and King, 1979) in the freeze preservation of *Zea mays*. Its use was prompted because of the cytotoxicity of conventional cryoprotectants, especially glycerol. It has been implicated in salt stress protection in halophytes and cold and desiccation stress resistance in higher plants. It has a very high solubility, is pH neutral (pH 7–7.5), exerts a high osmotic pressure and is non-toxic at high concentrations. Pre-growth for 3-4 days in basal medium incorporating 10% (w/v) proline increased post-thaw viability from 20 to 60%.

(c) Plant Growth Regulators- Pre and post cryopreservation

A third approach via pre-culture to improve the species explant cryotolerance is to incorporate plant growth regulators into pre-culture medium (Table 1.4). There is to date no general review available on the roles and use of plant growth regulators both as a pre- and post cryopreservation aid and there is certainly a need for such information. The general trend in the literature suggests the incorporation of ABA is useful as a pre-treatment desiccant in some species especially when co-ordinated with a cold treatment. The cytokinins, kinetin and zeatin; the auxin IAA and other hormones such as GA_3 have been shown to promote post-thaw recovery. Thidiazuron (TDZ) is among the most active cytokinin-like substance for woody plant tissue and has been used to facilitate micropopagation of many recalcitrant

woody species (Huetteman and Preece, 1993) and may assist in the post-cryopreservation recovery of Sitka spruce shoot cultures.

ABA as a pre culture plant growth regulator

Increased ABA concentrations in cells have been suggested to trigger cold acclimation and expression of low temperature responsive genes. ABA has been shown to increase the cold hardiness of cell cultures and whole plants of many species (Chang and Reed, 2001). The temperature at which 50% of cultures die (LT_{50}), is routinely used to compare the effectiveness of cold hardening treatments. *In vitro* grown pear shoots cultured with alternating low temperature treatments and 150μM ABA showed a 12°C lower LT_{50} than untreated controls.

Post-cryopreservation plant growth regulators

It has been observed in many species that plantlets derived from cryopreserved germplasm often exhibit high survival in the first 1-2 wks of post-thaw recovery but then show senescence in the following two to four weeks, and this may be due to sub-optimal recovery conditions. Turner, *et al.*, (2001b) working with *in vitro* shoot cultures of the rare Australian plant species *Anigozanthus*, demonstrated that post-thaw plantlets treated with 0.5μM kinetin/ 0.5μM GA_3 showed a mean increased length of 4.3mm, compared with the untreated controls. Cytokinins stimulate cell division, reduce apical dominance and control morphogenesis. GA_3 causes internode elongation and plays a vital role in seed germination.

Table 1.4 Plant growth regulators incorporated into pre-and post cryopreservation regimes.

Plant species	Explant	Pre-cryo	Post-cryo	Recovery/ abnormalities	References
Vanda pumilia (Orchidaceae)	Shoot apices *in vitro* protocorms	3 –days 1.0mg/l ABA	10 days recovery then 0.02mg/l BAP	No abnormalitics in chromosome number or cell structure	Na and Kondo, (1996)
Betula pendula	*In vitro* shoot tip apices	28 days 10^{-4}M ABA + CH	-	Increased callus formation from post-thaw shoots	Ryynanen, (1998)
Pyrus cordata	*In vitro* shoot tip apices	3 weeks 50μM ABA + CT	-	Shoot tip growth from 0-70%	Chang and Reed, (2001)
Anigozanthos viridis ssp *terraspectans*	*In vitro* shoot tip apices	-	28 days post thaw from day 0. *Cytokinins* 1. Kinetin 2. Zeatin *Auxin* 1. IAA 2. GA_3	All improved recovery from control	Turner, *et al.*, (2001b)

CH= Cold treatment; IAA= Indole-3-acetic acid; GA_3=Gibberelic acidcryopreservation active cell growth to produce organised tissues rather than undifferentiated callus.

Thidiazuron (TDZ)

Thidiazuron has a tremendous ability to stimulate shoot proliferation in a wide array of woody plant species (Huetteman and Preece, 1993). Thidiazuron is one of several substituted ureas investigated for cytokinin activity. TDZ was developed originally as a defoliant for cotton, but was later discovered to have an auxin function as well. In small doses (<1 μM) it induces greater axillary proliferation, especially in woody species explants, than many other cytokinins. At concentrations higher than 1 μM, callus, adventitious shoots or somatic embryos may be formed (Huetteman and Preece, 1993). In some cases fasciated shoots may be formed. TDZ shows promise for *in vitro* woody plant manipulations and will be applied to *P. sitchensis* shoot cultures as a component of the post-thaw recovery culture.

1.4.4.2 Cryoprotectants

Cryoprotectant is a functionally-derived term to describe, 'any additive which can be provided to cells before freezing and yields a higher post-thaw survival than can be obtained in its absence,' (Fuller, 2004). A number of successful cryopreservation protocols of biological material depend upon the addition of exogenous cryoprotective compounds. In the 1890's Hans Molisch became aware that the composition and concentration of substances in the plant cell cytoplasm dictated survival or death (Stout, 1982). The importance of sugars as cryoprotectants was recognised by Maximov in the early 1900s. By the 1950s small molecular weight solutes with high aqueous solubility such as glycerol, were applied to cryopreservation in medicine, biotechnology, plant and animal breeding (Fuller, 2004).

a) Modes of protection

Cryoprotectant solutions can be sub-divided into 3 modes of action; penetrating, non-penetrating and combinations depending on their permeation of the cell plasma membrane.

1) *Non-penetrating polymeric cryoprotectants*:

High molecular weight compounds are unable to penetrate the cell membrane but act to osmotically dehydrate the cell, thereby reducing the amount of cellular water available for intracellular ice formation (Benson, 1990). Examples include sucrose, trehalose and sorbitol (which in some cases may be actively taken up by cell and metabolised) and polyethylene glycol.

2) *Penetrating colligative cryoprotectant*

Small, low weight compounds are thought to protect the cell in a number of ways:

(a) by lowering the temperature at which the intracellular water freezes (Kartha, 1985)
(b) their colligative action, reduces the concentration of damaging solutes and increases the unfrozen fraction, thereby limiting deleterious volume changes (Benson, *et al.*, 2005 In press)
(c) by kinetically inhibiting or retarding ice crystal growth through the viscosity of the cryoprotectant solution (Fuller, 2004)

Examples include proline, glucose and dimethyl sulphoxide (Me_2SO_4).

3) *Penetrating and non-penetrating cryoprotectant combinations*

Cryoprotectant vitrification cocktails (such as PVS2) incorporate mixtures of different cryoprotectant types: penetrating, such as glycerol (in some cell types) and DMSO; non-penetrating sucrose. In these cases, the addition of polymeric compounds assist by reducing

the required concentration of penetrating cryoprotectant and therefore avoiding toxicity (Finkle, *et al.*, 1985). The penetrating cryoprotectants that vitrify the most powerfully are those that hydrogen-bond strongly to water and interfere with ice formation via water to water hydrogen bonding (Fahy, *et al.*, 2004b). These cryoprotectants are also the most toxic because they bind strongly to proteins causing denaturisation.

b) Examples of cryoprotectants

Glycerol

Glycerol may or may not penetrate cells according to species and application conditions. Glycerol is a small poly-hydroxylated solute with a high water solubility and a low toxicity during short-term exposure to cells. It interacts by hydrogen bonding with water and can slowly penetrate the cell membrane of many cell types.

DMSO

DMSO is a widely employed plant, human and animal cell cryoprotectant because of its rapid penetration through cell walls and plasmalemma. Even in the relatively short exposure of PVS2 protocols (typically 30 min to 4hr), DMSO is considered to penetrate the cytosol. There are several theories as to why the particular properties of DMSO make it such an effective cryoprotectant, and what the precise mechanisms are that it utilises. These include:

(1) DMSO as a vitrification cryoprotectant prevents ice formation by weak hydrogen bonding and through colligative interference of ice formation (Fahy, *et al.*, 2004a, Fahy, *et al.*, 2004b).
(2) Protein stabilisation by preferential exclusion (Arakawa, *et al.*, 1990). DMSO is part of a group of cryoprotectants that are preferentially excluded from the hydration shell of proteins at low temperatures; this exclusion is believed to lead to thermodynamic stability during cooling. Paradoxically such compounds are toxic to proteins at higher temperatures (see section *c* below).
(3) DMSO may also facilitate the entry of other cryoprotectant components for the protection of intracellular membranes; such as trehalose (Beattie, *et al.*, 1997).
(4) Good thermally stable glass–forming tendencies (even at low concentrations) (Kim, *et al.*, 2004).

(c) Balance of toxicity

Cryoprotectants such as DMSO and ethylene glycol may be toxic to biological systems especially at room temperatures where they are known to denature enzymes.

Evidence in higher plants and from micro-organisms and animal cells indicates that DMSO in 2 to 10% solution may be involved in generating a variety of genetic and /or epigenetic changes (Finkle, *et al.,* 1985).

Arakawa, *et al.*, (1990) offered a 'dual temperature–dependent mode' explanation for apparent cryoprotectant toxicity. Cryoprotectants such as DMSO and ethylene glycol stabilise proteins during freezing but destabilise them at physiological temperatures. At low temperatures these cryoprotectants are preferentially excluded from the hydration shell of the proteins and this exclusion leads to stabilisation. It is suggested that proteins are destabilised at higher temperatures as co-solvents interact hydrophobically with proteins. The acquisition of balance between cryoprotection and toxicity damage is key in cryopreservation protocol optimisation and will form a key part of *P. sitchensis* germplasm cryostorage development.

1.4.5 Development of cryopreservation protocols

There are a number of factors to consider in the development of cryopreservation protocols both in biological suitability and operator practicality. Some explants and genotypes may exhibit specific desiccation or toxicity sensitivity and the freezing technique will be largely dictated by recovery rates (Benson, 1999a). In large-scale genebanking controlled rate cooling may be the most preferential approach because the simultaneous freezing of large sample numbers is cost efficient.

(a) Controlled rate cooling

Controlled rate cooling allows specific control of cell dehydration by controlling the rate and hold temperatures. At the point of optimum dehydration slow freezing is usually terminated at ca. -40 °C to minimize ice crystal formation from remaining intracellular water. Controlled rate cooling systems first incorporated the use of a cold immersion bath (filled with solvent such as industrial methylated spirits) set at an intermediate temperature (-30°C) to cool samples prior to LN immersion. Withers and King, (1980) developed a successful protocol to cryopreserve cell suspension cultures of *Acer*, *Zea* and *Rosa*. The freezing unit consisted of a dip-cooler immersed in a methanol bath. Cell suspensions were cooled slowly to -35°C and held for 30min prior to liquid nitrogen immersion. These methods were the basis for the development of the programmable rate freezer and 'Mr. Frosty™' units (Benson, 1999a).

Programmable rate freezers

A programmable freezer is a commercial instrument (Fig.1.8), comprising a freezing chamber into which liquid nitrogen is supplied from a pressurized Dewar controlled by a solenoid

valve. Samples may be placed in a chamber constructed to hold cryovials or straws. A computer software link enables the programmer to specify a range of temperature rates, ramps and start and finish parameters. These parameters are critical to success and may be species/explant specific. Following controlled cooling to a terminal transfer temperature, cryovials or straws are transferred to liquid nitrogen.

This method of freezing has been the most successful in the cryopreservation of conifer embryogenic cell suspensions (Table 1.1). Find, *et al.*, (1993) successfully cryopreserved embryogenic suspension cultures of *P. sitchensis*. This was achieved using a 0.4M sorbitol pre-treatment, 5% (v/v) DMSO cryoprotection and cooling in a controlled rate freezer, at a rate of -0.5°C/min, to a terminal temperature of -40°C/min, followed by transfer to liquid nitrogen.

Figure 1.8
Planar KRYO SERIES III Programmable Freezer 20-30 canes – 3 cryovials per cane 60-90 cryovials per cooling run

Mr. Frosty™ Nalgene

A second controlled rate freezing apparatus (Fig. 1.7) permits cryovial cooling for typically 1 hr in a fixed temperature freezer (-80°C). The unit consists of a high-density polyethylene container containing 100% isopropanol and is trademarked as "Mr. Frosty" (by Nalgene®). Mr Frosty™ provides a cooling rate of –0.99°C /min (Cyr, 1999) and is 86*117mm (height x diameter), easily transported and can reduce costs as it circumvents the need for expensive controlled rate programmable freezers. 'Mr. Frosty,' has been adopted for routine use for a number of conifer species by the Canadian Forest Service (Dr. Yill Sung Park, pers. comm).

Figure 1.7
Mr. Frosty ™ polyethylene container (20 cryovials)

(b) Rapid Cooling

Rapid cooling is based on the principles of vitrification whereby ice formation is circumvented and amorphous glass is produced:

Encapsulation-dehydration

Fabre and Dereuddre, (1990) first encapsulated *Solanum* shoot-tips into Na-alginate beads, osmoprotected them in liquid medium enriched with 0.75M sucrose for 1 to 2 days, before laminar air-flow desiccation to a water content around 20% (FW) prior to LN immersion. The processes of progressive sucrose osmotic dehydration and laminar air-desiccation lead to sucrose saturation in the bead resulting in a glass transition upon cooling to -196°C. Advantages of this technique include the elimination of cryoprotectant application, other than sucrose, ease of handling encapsulated explant and reduction in pre-treatment operation time compared to chemical vitrification. So far this technique has been applied to cryopreservation of woody plants explants, shoot-tips, ESM and somatic embryos including those shown in Tables 1.1 and 1.2.

Plant Vitrification Solution 2

Chemical vitrification requires a highly concentrated solution, which sufficiently dehydrates cells without injury to promote stable amorphous glass formation when immersed into LN. Sakai, *et al.*, (1990) developed a glycerol-based, low-toxicity vitrification solution termed PVS2. This solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO in MS basal medium containing 0.4M sucrose (pH 5.8). In encapsulation-vitrification protocols, samples are encapsulated in alginate beads, osmoprotected in sucrose and/or glycerol and then dehydrated in PVS2/PVS3 for 0.5-3hr prior to plunging in LN (Hirai and Sakai, 1999, Matsumoto, *et al.*, 1995, Tannoury, *et al.*, 1991).

The protocols have been applied successfully to a variety of species and explants including roots and tubers, fruit trees, ornamental and medicinal plants and plantation crops. Examples of PVS2 and PVS3 application to woody plant species are shown in Table 1.2.

1.4.6 Post-cryopreservation viability testing

Viability tests are usually applied in cryopreservation investigations, but to date no single viability test has been developed that is suitable for all types of plant material under all experimental conditions. Distinguishing between living and nonliving tissues remains a difficult task (Calkins and Swanson, 1990). Several methods of tissue viability testing are based on the premise that freezing causes membrane damage.

(a) Observational methods

Macroscopic and microscopic examinations often reveal symptoms of freezing injury as a water-soaked appearance, tissue discolouration (especially browning) and reduced re-growth capacity. Cell organisation, distortion and lack of protoplasmic streaming or cell division may indicate injury or death, (Parker, 1953a).

(b) Vital Staining

Vital stains usually penetrate and are visualised in living cells preferentially to nonliving cells (Calkins and Swanson, 1990) dependent upon the basis of the test. A dehydrogenase-reduced salt, 2,3,5-triphenyltetrazolium chloride (TTC), has been used to determine woody plant cold hardiness (Parker, 1953b). TTC is a water-soluble, colourless salt that may be reduced by the dehydrogenase enzyme systems of living cells to form triphenyl formazan, a red insoluble product. TTC has several advantages over other enzyme tests: (1) it is non-toxic to humans (2) it will not diffuse out of cells once reduced (3) only a small amount of tissue is required. Disadvantages off TTC as a viability tests include: (1) rapid reduction and degradation in light; (2) may be reduced by reducing sugars, glutathione, ascorbic acid and cysteine at a pH above 9; (3) the enzymes that reduce TTC may function *in vitro*, thus dead cells may show a positive response. TTC has been applied to *Picea* somatic embryos as a post-drying and freezing viability test, but tended to overestimate freezing tolerance (Percy, *et al.*, 2001).

Another group of substances that may be used for vital staining techniques are those that fluoresce under ultraviolet light. The fluorescein diacetate vital stain is dependent upon the ability of esterases present in viable cells to cleave the stain, which then fluoresces yellow/green under a UV microscope (Widholm, 1972). Endogenous, fluorescent substances in plants such as chlorophyll a, b and riboflavin may complicate florescent dye interpretation. FDA viability was selected as a post-thaw test for several conifer suspension culture trials including *Pinus roxburghii* (Mathur, *et al.*, 2003) *Larix x eurolepis* and *P. mariana* (Klimaszewska, *et al.*, 1992). Protocols typically involve dissecting small sections of tissue (20-30mg), placement on a glass slide and adding 0.05% (w/v) FDA in liquid medium solution for a few minutes before examination under a UV microscope.

Other methods that may be used for viability testing include (Calkins and Swanson, 1990): (1) plasymolysis and deplasmolysis investigation in hypotonic sugar and/or salt solutions;(2) chlorophyll fluorescence direct or using chloroplastic lipids and proteins as intrinsic probes for environmentally induced change, (3) electrical conductivity, (4) organic material leakage, and (5) gas exchange.

Vital staining should not be used alone to estimate the survival of embryos. Cells may be in a state of 'cryo-shock', and may not show signs of viability immediately, but after an extended period of culture (even after 6mths), they may resume growth. Survival data may be determined as embryo cultures show an: (1) increase in size, (2) greening, (3) proliferation of callus, (4) development of plantlets.

Viability stains indicate cell recovery and injury following cryopreservation but they provide no direct information as to the cause of injury or death. Cryopreservation may be monitored physically by thermal analysis techniques such as differential scanning calorimetry (DSC).

1.4.7 Thermal analysis– differential scanning calorimetry

DSC measures the heat-flow during a preset (10°C/min) cooling and warming cycle, between two pans (the sample tissue was sealed e.g. in an aluminium pan, against the control reference pan) and the thermal difference (mWatts) is plotted against time, or temperature, to produce a thermogram (Benson, *et al.*, 2005 In press) . Ice formation (nucleation), re-crystallisation, melting, de-vitrification; and glass formation/relaxation all produce thermal events that alter the heat flow required to maintain both pans at the same temperature. This causes a change in the heat flow manifested as an exothermic, or endothermic event, resulting from the latent heat of fusion of water. Thermograms, during cooling show: (1) peaks in water ice transitions and (2) deflections from the baseline in water amorphous glass transitions (Tgs). The thermograms produce, a great deal of information, about the water status in samples and can show the effectiveness of treatments, for example, if ice is circumvented, in a cryopreservation protocol or if further optimisation is required.

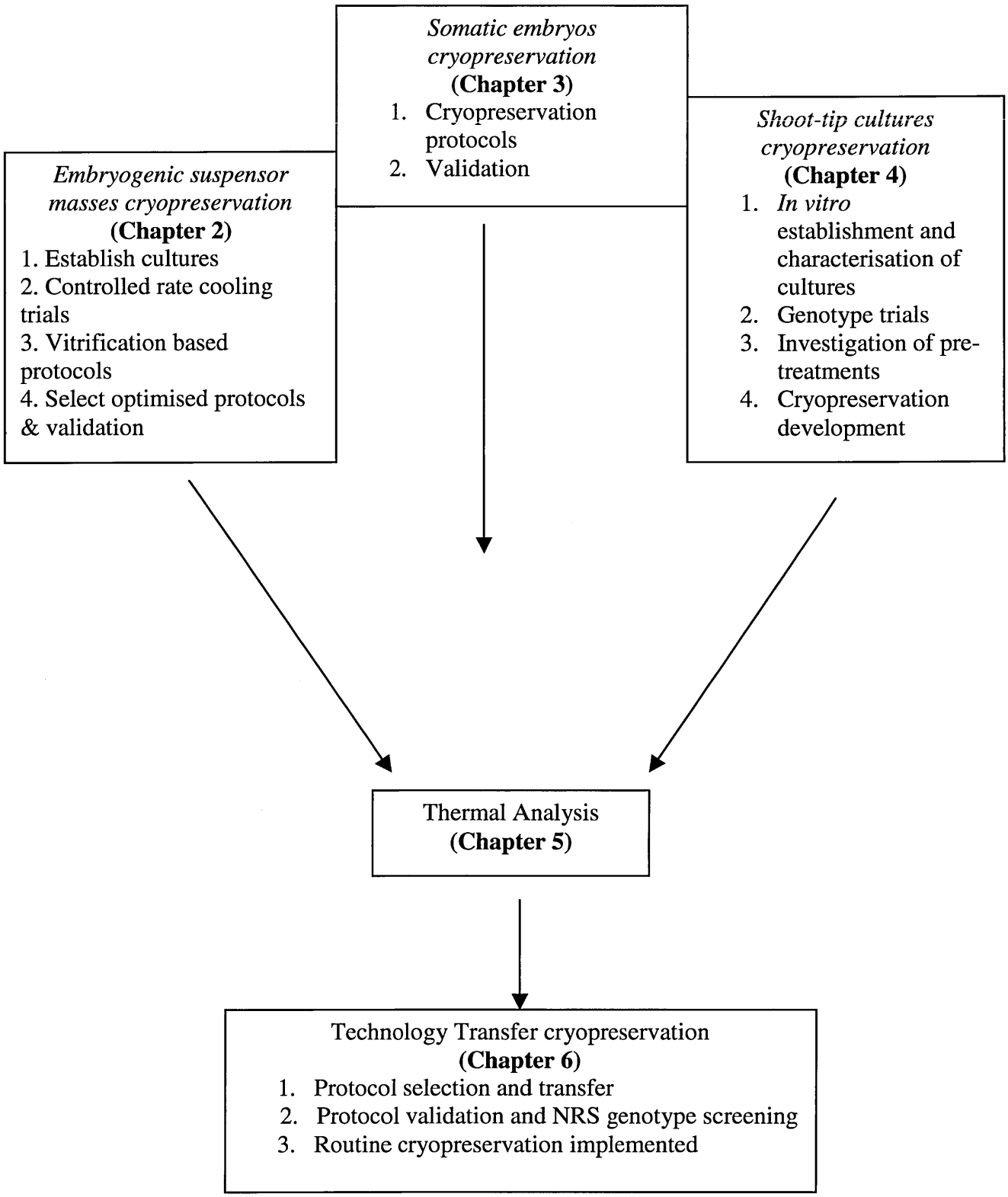
Examples of differential scanning calorimetry

Examples of DSC application include: (1) studies on *in situ* lateral buds of Douglas-fir (*Pseudotsuga menziesii*) seedlings in both non-hardy and hardy conditions and how to detect low-temperature exotherms and nucleation of supercooled aqueous fractions indicative of lethal freezing in certain plant tissues (Stushnoff, *et al.*, 1992), (2) encapsulated cryopreserved carrot somatic embryos (Dereuddre, *et al.*, 1991), (3) encapsulated-dehydrated suspension cells of *Arabadopsis thaliana* (Bachiri, *et al.*, 2000) and *Catharanthus roseus* (Bachiri, *et al.*, 1995).

1.5 Study rationale

This thesis will investigate the application of cryopreservation to *P. sitchensis* germplasm by applying fundamental analytical techniques to interpret the success or failure of different protocols. Discussion as to the critical points in technology transfer and pilot genebank establishment will be an integral part of the study. The thesis is structured into 7 chapters (Fig. 1.9). The success and efficiency of a range of cryopreservation methods as applied to 3 explant types is discussed: embryogenic suspensor masses (Chapter 2); somatic embryos (Chapter 3) and shoot-tip apices (Chapter 4). The thermal stability of a selection of vitrification protocols of each of the explants is explored in Chapter 5. The technology transfer recommendations and establishment of a pilot genebank are discussed in Chapter 6. The final discussion (Chapter 7) will summarise the findings, recognise trends between investigative chapters, draw conclusions and make future recommendations.

Figure 1.9 Thesis structure showing the integration of applied and fundamental components



Chapter 2 CRYOPRESERVATION OF EMBRYOGENIC SUSPENSOR MASSES

2. Introduction

The objective of this chapter is to develop a robust cryopreservation protocol, for spruce embryogenic suspensor masses (ESM). This will be achieved through the optimisation of: pre-treatment, cryoprotection and the screening of traditional, slow, (controlled rate) cooling and vitrification protocols. The most effective protocol will be selected, optimised further and validated over 3 years during which time it will be applied to 25 embryogenic *P. sitchensis* cell lines. Further objectives will be to assess embryo maturation potential in cultures recovered from cryogenic storage as well as the effects of genotype and prolonged *in vitro* storage on cryotolerance.

2.1.1 Controlled rate cooling methodologies

Controlled rate or slow cooling protocols rely on controlling ice nucleation, extracellular ice formation, the rate of cooling and colligative cryoprotection, so that the two factors involved in cryoinjury, ice and dehydration, are not lethal to the cell (see section 1.4.5 in Introduction). Find, *et al.*, (1993) successfully cryopreserved embryogenic suspension cultures of *P. sitchensis*. This was achieved using a 0.4M sorbitol pre-treatment, 5% (v/v) DMSO cryoprotection and cooling in a Controlled Rate Programmable Freezer, at a rate of -0.5°C /min, to a terminal temperature -40°C /min, followed by transfer to liquid nitrogen (LN). This protocol was originally developed for homogeneous cell suspension cultures. In the study, the protocol will be adapted to cryopreserve differentiated, heterogeneous embryogenic suspensor masses (ESM).

A second controlled rate cooling protocol used in this study utilized a simplified controlled rate cooling apparatus, which permitted cryovial cooling for typically 1 hr in a fixed temperature freezer (-80°C). The unit consists of a high-density polyethylene container containing 100% isopropanol and is trademarked as "Mr. Frosty" (by Nalgene®). Mr Frosty™ provides a cooling rate of –0.99°C/min (Cyr, 1999) and is 86*117mm (height*diameter), easily transported and can reduce costs as it circumvents the need for expensive controlled rate programmable freezers. Mr Frosty™ has been adopted for routine use for a number of conifer species by the Canadian Forest Service (Dr. Yill Sung Park, pers. comm.).

2.1.2 Vitrification methodologies

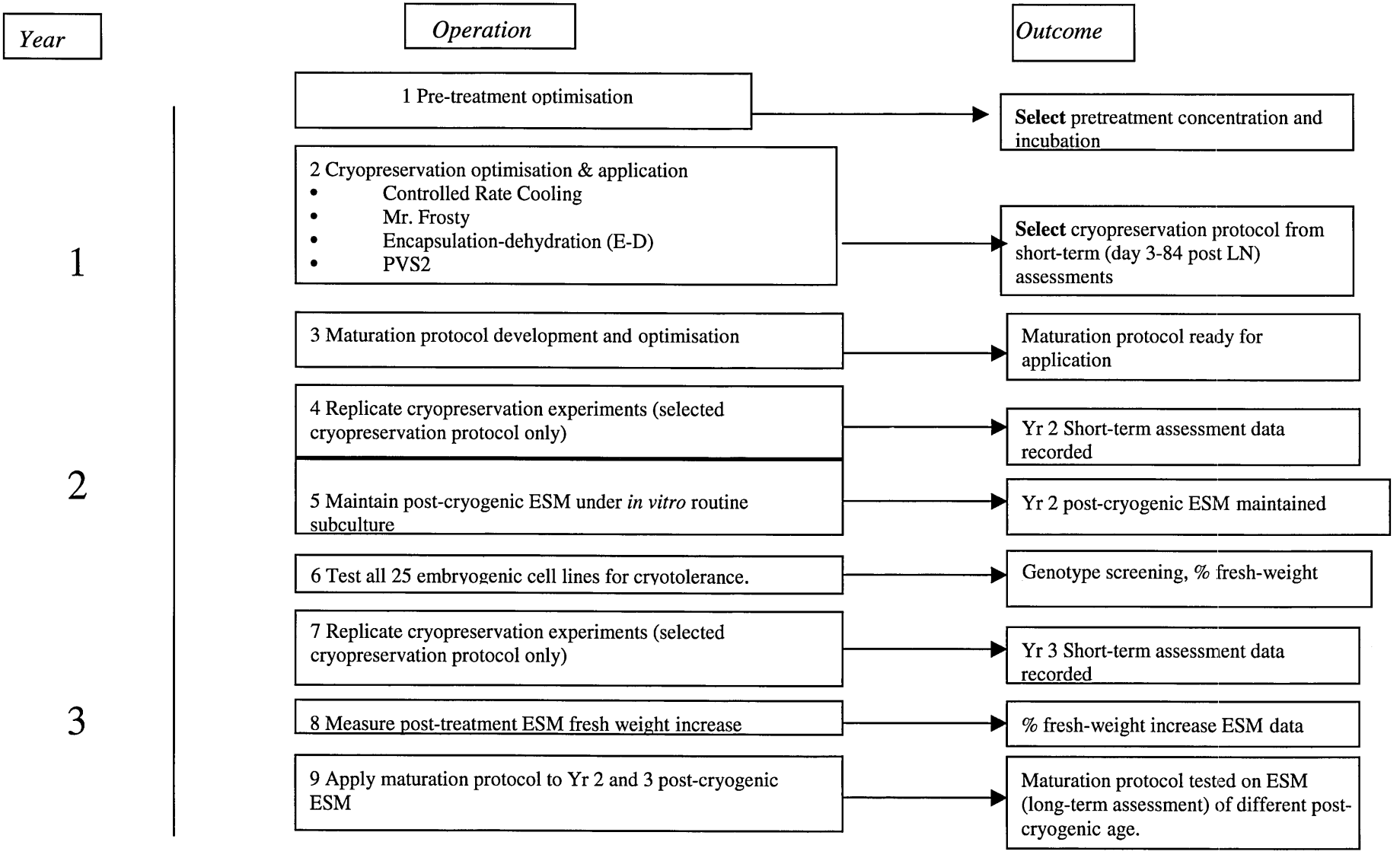
Vitrification protocols rely on circumventing ice formation, and instead a glass transition (Tg) from water to an amorphous 'glassy' state occurs. This is achieved by manipulating solute concentration (e.g. by desiccation or chemical osmotica) such that cell viscosity becomes sufficiently high so as to inhibit ice crystal formation (see section 1.4.3 in Introduction). Encapsulation-dehydration protocols consist of explant encapsulation in a Na-alginate bead, osmotic dehydration, desiccation and LN immersion. They are based on the founding work of Fabre and Dereuddre, (1990). There are no reported applications of cryopreservation in ESM via encapsulation-dehydration in conifers, but protocols have been developed for embryogenic cells of woody plant species; grapevine embryogenic cell suspensions (Wang, *et al.*, 2002); and mango embryogenic masses (Wu, *et al.*, 2003). Plant Vitrification Solution (PVS) based-protocols vitrify plant tissue using high concentrations of penetrating (e.g. DMSO) and osmotically active, dehydrating cryoprotective additives. Plant Vitrification Solution 2 (PVS2) consists of a highly concentrated cocktail of ethylene glycol, DMSO and glycerol and was developed by Sakai for nucellar cells of naval orange (Sakai, *et al.*, 1990). PVS2 cryopreservation was successfully applied to ESM of *Picea mariana* (Touchell, *et al.*, 2002).

2.2 Materials and Methods

2.2.1 Experimental Design

The experimental strategy is described in Figure 2.1. In Year 1, 5 representative genotypes of ESM were selected from 25, and used to optimise pre-treatments and test cryopreservation responses in four freezing protocols. A protocol was selected based on the results of this preliminary study and short-term assessments undertaken, this was repeated for Years 2 and 3. During this time an embryo maturation protocol was also developed and optimised in association with the Forestry Commission's Northern Research Laboratory, Roslin, Edinburgh. In Year 2 the optimised cryopreservation protocol was selected and applied to the remaining 20 genotypes. Post- cryogenic ESM cultures from some genotypes derived from Years 2 and 3 were maintained under *in vitro* subculture. In Year 3 all post-cryogenic ESM cultures underwent embryo maturation testing and long-term culture health assessments were applied.

Figure 2.1 Experimental Design for Embryogenic suspensor mass (ESM) Cryopreservation



2.2.2 Establishment of ESM lines

Developing cones were collected in July 1998 from five open pollinated clones in a clone bank in Perthshire. The five progeny tested clones were Sitka spruce SS394 (A), SS492 (B), SS1102 (C), SS1615 (D) and SS551 (E). Immature embryos were excised from the developing seeds at Northern Research Station (NRS), Roslin, Edinburgh and cultured on Somatic Embryogenesis Induction Medium (SEIM) containing 0.43mg/l kinetin, 11.5mg/l 2,4-dichlorophenoxyl acetic acid (2,4-D) and 0.45mg/l 6-benzylaminpurine (BAP) (John, *et al.*, 1995). SEIM also included 15g/l of sucrose and 6g/l agar at pH 5.7.

Embryogenic cell lines were transferred to Somatic Embryogenesis Maintenance Medium (SEMM) as before, but at a reduced level of plant growth regulators (PGRs) 1.105mg/l 2, 4-D levels (John, *et al.*, 1995). Embryogenic cell lines were subsequently maintained in soda glass tubes (Oxoid™ 100mm (ht) x 25mm (diam.)), sealed with polyethylene cling film and sub-cultured every 4 weeks. Cultures were incubated in the dark at 20 $\pm$ 1°C and were approximately 3 years old at the start (2001). ESM cultures of five cell lines, each of five open-pollinated families (i.e. twenty-five cell lines in total) selected for the cryopreservation study, were designated A-E and were transferred from the Northern Research Station Laboratory, Roslin, to UAD one month before the commencement of cryopreservation studies. Thereon they were maintained in 50mm Sterilin™ Petri plates and sealed with Parafilm to facilitate cryogenic treatments. One genotype from each family was selected (on a fresh weight increase basis, for vigour) to aid cryopreservation protocol optimisation and selection procedures. These were designated as lines A5, B1, D3, C3 and E3.

2.2.3 Embryo Maturation

Embryo maturation studies were undertaken at the Northern Research Station, Roslin using genotypes selected on the following criteria: representation of each genetic group, significance for Forestry Commission breeding programmes and culture vigour. Cultures were maintained for 6 weeks on SEMM before transfer to Somatic Embryogenic Activated Charcoal Medium, SEAC, (10g/l, 25mls per plate, pH 5.7) without plant growth regulators for 1 week (John, et al., 1995). The cultures were then transferred to Somatic Embryogenic Abscisic Acid Medium, SEABA (John, *et al.*, 1995) containing 25mg/l abscisic acid (ABA) for 6-10 weeks.

Post-cryogenic ESM was selected from cultures re-grown from experiments undertaken in Years 2 and 3. The ESM was selected at the second subculture cycle, pooled from 3 recovery Petri plates, transferred to soda glass tubes, (10 replicates) and maintained on SEMM under standard culture conditions. Cultures maintained at the Northern Research Station for 5 years were used for comparison and designated as non-cryopreserved controls of the same genotypes, studied at the same age.

Morphogenic competence was assessed after 10 weeks of culture on SEABA (Fig. 2.2). In this time, culture health and the development of stage 1 embryos to stage 6 emblings (plantlets that have fully differentiated structures and needle primordia) was monitored. Culture health was assessed as the increase in ESM volume, measured as the mean of two diameters of the ESM, taken at 90° to each other. There were 5 tubes for each treatment and genotype and the experiment was repeated twice. Data was analysed as mean diameter $\pm$ SEM for each non-cryopreserved and cryopreserved genotype. The Student's paired T-test was used to compare non-cryopreserved (control) and cryopreserved pairs of each genotype. Different letters will be used to denote groups if cryopreserved pairs show statistically different means at $P<0.05$. The minimum number of embryos per tube at each developmental stage was also assessed. The mean $\pm$ SEM of the 10 replicates (tubes), 5 per experiment, is presented for each genotype and treatment.

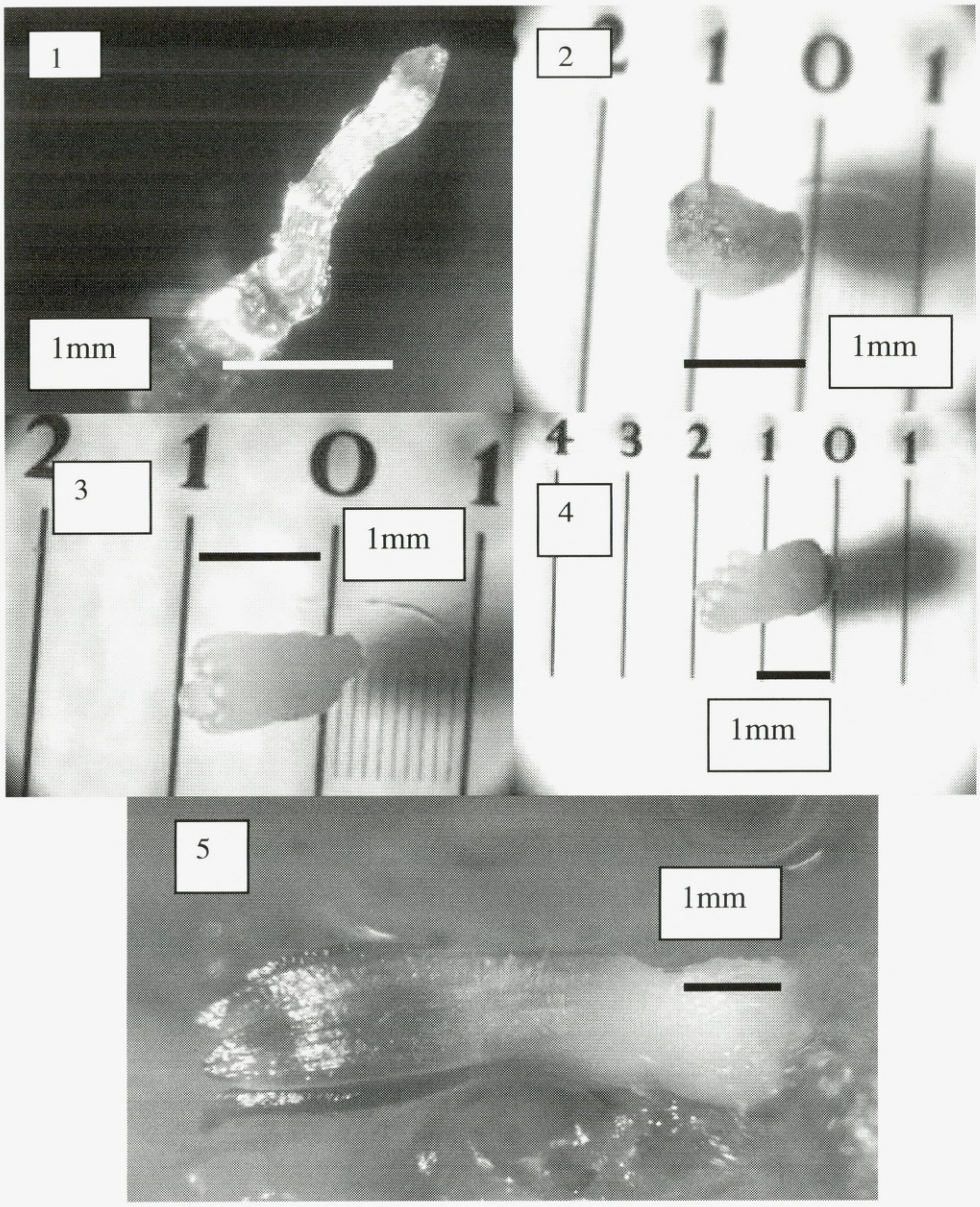


Figure 2.2 - Stages of embryo development: 1. embryo development (a differentiating ESM showing early polar primordial development, size range 0.5mm X 0.5-1.0mm); 2 and 3-globular/heart-shaped, size range 0.5-1.0 X 0.5-1.0mm; 4 torpedo with polar meristems, size range 0.5-1.0 X 1.0-1.2mm; 5 cotyledonary with chlorophyll forming in extending shoot primordial, size range1.0-1.5 X 2-2.5mm

2.2.4 Sorbitol pre-treatment optimisation

Pre- culture optimisation of sorbitol pre-treatment was undertaken and all ESM tested for pre-treatment optimisation and cryopreservation were transferred from SEMM at day 14 of a 28-day culture cycle. In experiment 1, 1g of ESM, genotype E3, was transferred to SEMM incorporating 0.4-1M of sorbitol. After 48 hours on sorbitol under routine conditions (Section 2.2) ESM was transferred back to SEMM for 28 days. In experiment 2, ESM was cultured on SEMM incorporating 0.4M sorbitol for 2-10 days under routine culture conditions. ESM was then transferred back to SEMM for 28 days. The selected optimised pre-treatment method was subsequently applied to further cryopreservation experiments.

In both sorbitol pre-treatment experiments, 5 individual plates (1 ESM per plate) were tested and the experiment repeated twice. Percentage fresh-weight gain measurements were determined at day 28. In experiment 1, statistical analysis consisted of a 1-way ANOVA and Fishers Pair-wise comparisons and in experiment 2 (data not normal transformed log^{10}) a 1-way ANOVA and Fishers Pair-wise comparisons.

2.2.5 Cryopreservation

2.2.5.1. Controlled rate cooling

Cryoprotectant and freezing profile trials were initiated to optimise cryoprotectant composition and ice-nucleation temperature. Find's protocol (1993) incorporated 5% (v/v) DMSO was compared with a second cryoprotectant (Withers and King, 1980) incorporating 0.5M DMSO + 0.5M glycerol and 1M sucrose. Batches of 30ml volumes of cryoprotectants were prepared on the day of cryopreservation as described below:

Cryoprotectant 1 5% (v/v) DMSO cryoprotectant (30mls batch) was formulated by adding 1.5ml of DMSO to 28.5ml liquid SEMM media and buffering the cryoprotectant to pH 5.7.

Cryoprotectant 2 This was based on the original cryoprotectant developed by Withers and King, (1980). A cryoprotectant solution was developed containing; 0.5M glycerol, 0.5M DMSO and 1M sucrose. Glycerol is very viscous and therefore was added by weight (w/v) and not volume (v/v) to facilitate formulation. DMSO was added by volume and therefore corrected for volumetric addition using specific gravity, based on water to calculate mass and therefore molarity.

A typical 30ml batch was formulated with: 0.5M glycerol (1.38g), 0.5M DMSO (added by volume accounting for DMSO's specific gravity of 1.479; volume required was 0.795ml), and 1M sucrose (10.272g) prepared to a total volume of 30mls, using liquid SEMM. The formulated cryoprotectant was buffered to 5.7 (using 0.125M NaOH).

ESM was pre-treated on SEMM 0.4M sorbitol for 48hr, 0.5g tissue was transferred to 2ml cryovials (Simport™, self standing) and cryoprotected with filter sterilised, disposable filters (25mm diameter, sterile and non-pyrogenic, 0.2µm polyethersulfone membrane), cryoprotectant 1 or 2 and were added to a final volume of 1.8ml. Cryoprotection on ice was undertaken for 30 min; the vials were then transferred to cryo-canes. Cooling program for the Planar, UK (Programmable freezer KRYO 10 Series 111) was as follows:

		Program 1	Program 2
Ambient	=	0°C	0°C
Rate	=	-0.5°C	-0.5°C
Hold	=	None	-15 °C (10 min)
Finish Temp.	=	-50 °C	-50 °C

Cryocanes were transferred to the programmable freezer, and a temperature probe placed in one vial to compare thermal behaviour reproducibility in the vial between freezing runs. Five replicate cryovials (equivalent to ESM selected from 5 different plates) per experiment were tested and each experiment repeated twice. Each combination was assessed using qualitative assessments, vital staining FDA and quantitative assessment as % plate recovery. One genotype (A5) was initially tested and following optimisation, a full cryopreservation experiment was applied to 4 other genotypes. ESM was pre-treated and cryoprotected as optimised. Thawing, re-warming and recovery were conducted as described in section 2.2.5. Five cryovials (from ESM derived from 5 plates) per experiment were tested and each experiment repeated twice.

Vital staining FDA viability, % plates recovery and % ESM re-growth were recorded at each stage of the protocol. Percentage ESM re-growth data was not normally distributed and was transformed by $\log_{10}$ for statistical analysis (all graphical data is shown untransformed). The statistical significance of differences among treatments and genotypes at weighing days was analysed using a two-way ANOVA of repeated measures (S-Plus), $P<0.05$ was considered statistically significant. Statistically significant differences between treatments within each genotype were measured using Least Significant Difference (LSD), $P<0.05$ (not shown).

2.2.5.2 Mr. Frosty™

ESM was pre-treated and cryoprotected as optimised in section 2.2.4.1. The Mr. Frosty™ unit was filled with 100% isopropyl alcohol and placed on ice for 30 min to cool to 0°C. Cryovials containing cryoprotectant and ESM were transferred into the Mr. Frosty™ unit and transferred to:

1. the -20°C domestic freezer for 1hour

or

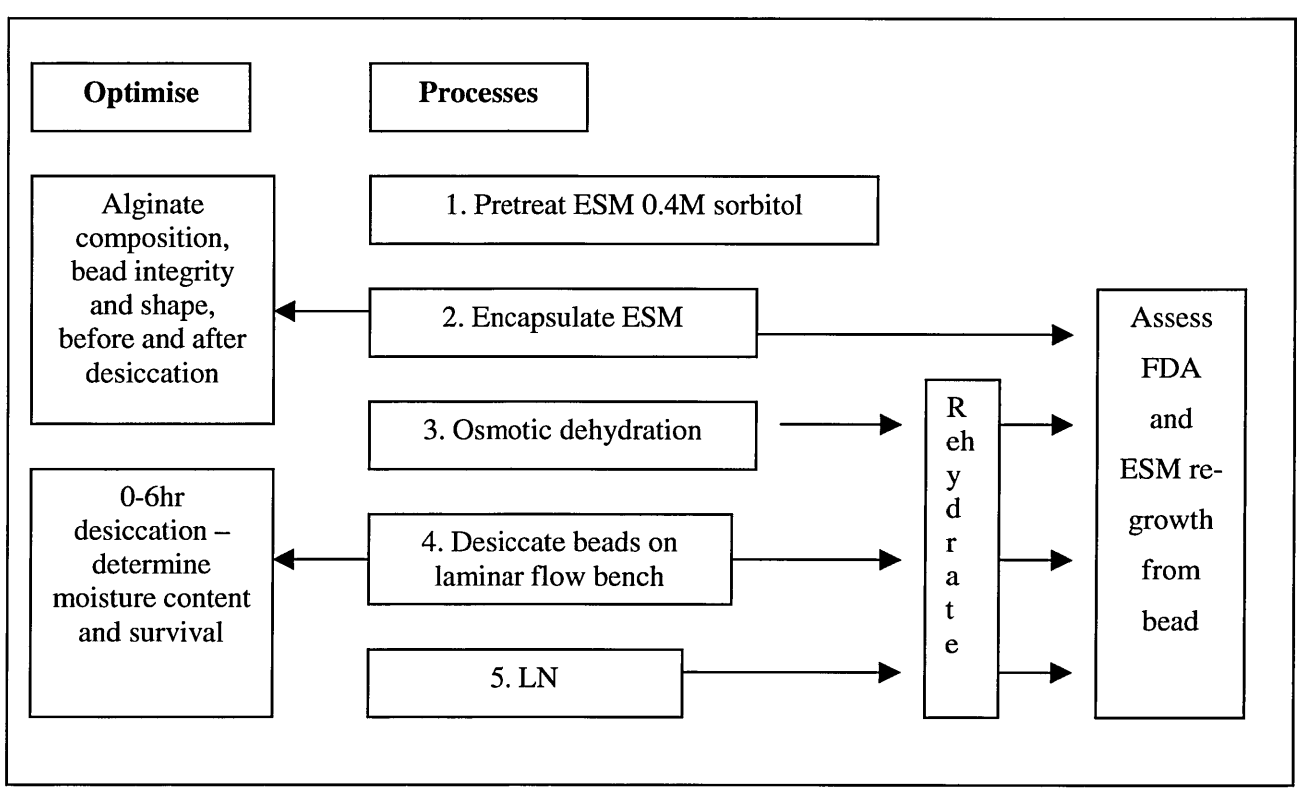
2. the -80°C domestic freezer for 1 hour

The cryovials were transferred to canes and then to liquid nitrogen for a minimum of 24 hr prior to thawing and recovery was as described in section 2.2.5. Five genotypes were tested, using vital staining FDA and % plate recovery to assess survival with and without LN immersion. Five replicate plates of ESM, per genotype, were tested, in one experiment.

2.2.5.3 Encapsulation-dehydration

Encapsulation-dehydration, based on the methods of Benson (1993) and Fabre and Dereuddre (1990), each step of the protocol was optimised for the *P. sitchensis* ESM cultures (Fig. 2.3). The concentration of sodium alginate and desiccation time were selected as critical factors.

Figure 2.3 Experimental optimisation and assessment of encapsulation-dehydration



ESM was pre-treated as optimised previously (Section 2.2.4). Alginate solutions were prepared by adding 3% (w/v) or 5% (w/v) sodium alginate (alginic acid low viscosity SIGMA A-21580) to liquid SEM (without $CaCl_2$). Solutions were agitated for 5 minutes prior to autoclaving and 10ml aliquots were dispersed into sterile 25ml Universal vials. Up to 0.5g of pre-treated ESM was added to each vial and a 3ml sterile-single Pasteur-plastic pipettes (Fisherbrand™) was used to submerge and disperse the ESM mass. Volumes in the range of 3mls of ESM and alginate were withdrawn and were dropped into another vial containing SEMM + 100mM $CaCl_2$ (care was taken to avoid air bubbles). Beads of ca. 4mm diameter were formed and left to polymerise for 20 min on a Petri plate. The shape of the beads was noted.

The beads were blotted dry on sterile filter paper and were transferred to another Universal vial (20 beads per vial) containing 0.75M sucrose in liquid SEMM. The vials were placed on a shaker (bench top, 120 rev/ min CERTOMAT ®) for 18hr (in the dark 20 $\pm$ 1°C).

Beads were poured onto a Petri plate, blotted on filter paper, placed on an open bottom Petri plate of 90mm diameter (Sterilin™), and then desiccated in a laminar flow bench (45% RH and 20°C) for 0-6hr. To determine the best desiccation time the moisture content (MC) of 10 beads was determined by weighing each bead immediately after sucrose incubation (T^0) and then after each hour (T^x) of desiccation (up to 6hr). After 6 hr the beads were placed on separate glass Petri plates (45mm diam.) and were placed in a 100°C oven for 12 hr (T^{dry}). Each bead was reweighed. The following formula was used to calculate the water content for each bead.

$$MC= ((T^x-T^{dry})/T^x)*100$$

Beads not used for dry weight analysis were desiccated and placed in a cryo vial (5 per vial), plunged into liquid nitrogen, re-warmed in 40°C water bath for 2 min and re-hydrated for 20 min in liquid SEMM. Re-hydrated beads were blotted on filter paper and transferred to solid SEMM (5 beads per plate). Beads were 3/4 immersed in medium. Beads were transferred to fresh medium every 2 weeks. Beads were examined for proliferating ESM through the alginate matrix, 42 days after treatment. Genotype A5 was used for FDA viability and ESM re-growth examination during desiccation optimisation. All assessment genotypes were tested for desiccation (time to be optimised) and cryopreservation recovery. Five replicate beads were tested per treatment in one experiment.

Plant Vitrification Solution 2

The PVS2 protocol was developed from Sakai's 2-step method (Sakai and Kobayashi, 1990, Touchell, *et al.*, 2002). ESM cultures were pre-treated as optimised previously (Section 2.2.4) and then 0.25-0.5g units of ESM were transferred to 2ml cryovials (Simport™, self-standing). The ESM was, 'loaded,' or osmoprotected with filter sterilised 2M glycerol and 0.4M sucrose (prepared in liquid SEMM), pH 5.8 at 0°C for 60 min.

ESM was transferred to freshly made pre-cooled (4°C for 1 hr), pre-filter-sterilised PVS2 solution (on ice for 30 min) comprising:
30% (w/v) glycerol
15% (w/v) DMSO
15% (w/v) ethylene glycol
0.4M sucrose,
The final solution was adjusted to pH to 5.8.

Cryo-vials were placed on cryo-canes with sleeves and immersed in LN for a minimum of 24hr. Vials were re-warmed, surface sterilised (Hibitane ™) and ESM was transferred to sterile Wilson sieves (100µm) pore size and rinsed with 1.2M sucrose made up in liquid SEMM at pH 5.8. Post-cryogenic re-warming and recovery was undertaken as described in section 2.2.5. ESM from 5 genotypes was used and 5 replicate cryovials (5 plates) were used per experiment, and the experiment was duplicated. The toxicity and cryogenic tolerance was examined through vital staining FDA and % plate recovery.

2.2.6 Re-warming and recovery

The cryovials were rapidly removed from LN and placed on a polystyrene float in a 40°C water bath for approximately 2 to 3 min. The vials were surface sterilised (Hibitane™), transferred to a flow bench and the contents emptied onto two layers of sterile filter paper discs (Whatman No.1, 45mm diameter) in Petri plates. The embryogenic tissue was drained and the top filter paper with tissue was placed on solid SEMM. On day 2, the tissue and filter paper were transferred to fresh medium and at day 7, transferred to fresh medium without the filter paper, transfers were repeated on days 14, 28 and 42. Short-term and long-term recovery assessments were undertaken using viability testing and fresh weight increase measurements, (Table 2.1).

Table 2.1 Recovery assessment–regimes applied to cryopreservation protocols (Years 1-3)

	Short-term assessments (3-84 days post-cryogenic storage)			**Long-term assessments (2-13 months, post-cryogenic storage)**	
Cryopreservation protocol and Year	Viability (FDA)	Viability (% plates showing recovering ESM colonies)	Fresh weight increase measurements per plate	Culture proliferation on maturation medium (diameter increase mm)	Embryo development (number per tube)
PVS2	✓	✓	✓	X	X
E-D	✓	✓	X	X	X
Mr. Frosty™	✓	✓	X	X	X
*Selection Yr1	✓	✓	X	X	X
*Selection Yr2	✓	✓	X	✓	✓
*Selection Yr3	✓	✓	✓	✓	✓

*Selected cryopreservation method applied
PVS2 = Plant Vitrification Solution 2; E-D = Encapsulation-Dehydration
FDA= Fluorescein diacetate; ESM= Embryogenic suspensor masses
✓= Assessment applied; **X**=assessment not applied

2.2.7 Short-term recovery assessments

Vital Staining Fluorescein diacetate

Fluorescein diacetate stain (Widholm, 1972) was used as a qualitative indicator only (due to highly heterogeneous structures of the ESM) and visualised as comparative viabilities in different morphological structures. A stock solution of 0.1% (w/v) FDA (Sigma) in acetone was stored at 4°C and 2-3 drops added to 20ml of sterile liquid SEMM. Embryogenic tissue was aseptically dissected to produce a cross-section of ESM 2-3 mm^2 and 0.5mm in depth, which was placed on a microscope slide. Two drops of FDA/SEMM solution were added and left for 1 minute before applying the cover slip to ensure the stain had penetrated the denser structures. The cells were examined with a DM/RB UV fluorescence microscope using a blue/violet filter without additional light. Qualitative examinations were undertaken to determine the % of fluorescing surface area. Three fields of view were examined per section and 3 sections per replicate plate were dissected. Images were captured using an RGB camera and Quantimet 550 hardware with Leica UK software.

% Plate recovery

Cultures were selected for % plate re-growth assessments at each treatment stage of the protocol. Untreated controls and pre-growth-treated cultures were directly transferred to solid SEMM; cryoprotectant controls and cryopreserved cultures were assessed after appropriate media-filter transfers. Visual assessments of ESM re-growth were undertaken using a

binocular microscope and the % is the proportion of plates showing proliferating ESM from the total number of recovery plates.

% Fresh weight increase

Quantitative fresh weights were determined for each treatment after 3, 14, 28 and 42 days of culture. The entire ESM contents on each plate were transferred (ensuring no medium or moisture excess on transfer) to a balance, weighed under aseptic conditions and transferred back to recovery plates.

2.2.8 Protocol selection and validation

The controlled rate cooling protocol, which yielded the greatest % plate recovery across the most genotypes chosen for preliminary investigation was selected for further testing.

2.2.8.1 Wider genotype screening

This protocol was then applied to a further 20 genotypes which had been maintained in *in vitro* culture in Year 2.

2.2.8.2 Long-term morphogenic studies

The optimised protocol was applied to the 5 genotypes initially selected in Year 1 for further study in Years 2 and 3. In addition, fresh weight analysis and embryo maturation studies were undertaken as described in section 2.2.3.

2.3 Results

2.3.1 Sorbitol pre-treatment optimisation

The response of ESM to varying sorbitol concentrations was monitored and all treatments applied to ESM showed and % fresh weight gain (Fig. 2.4 and 2.5), often greater than that of untreated ESM. Preliminary ESM examinations indicated that only a 1-2mm peripheral section of the ESM cluster encompassed viable cells. FDA analysis confirmed that the smaller cells (Fig 2.12) were present in this region, but a positive FDA response was observed throughout the cluster. ESM was therefore harvested from a cross-section of cell planes in subsequent experiments.

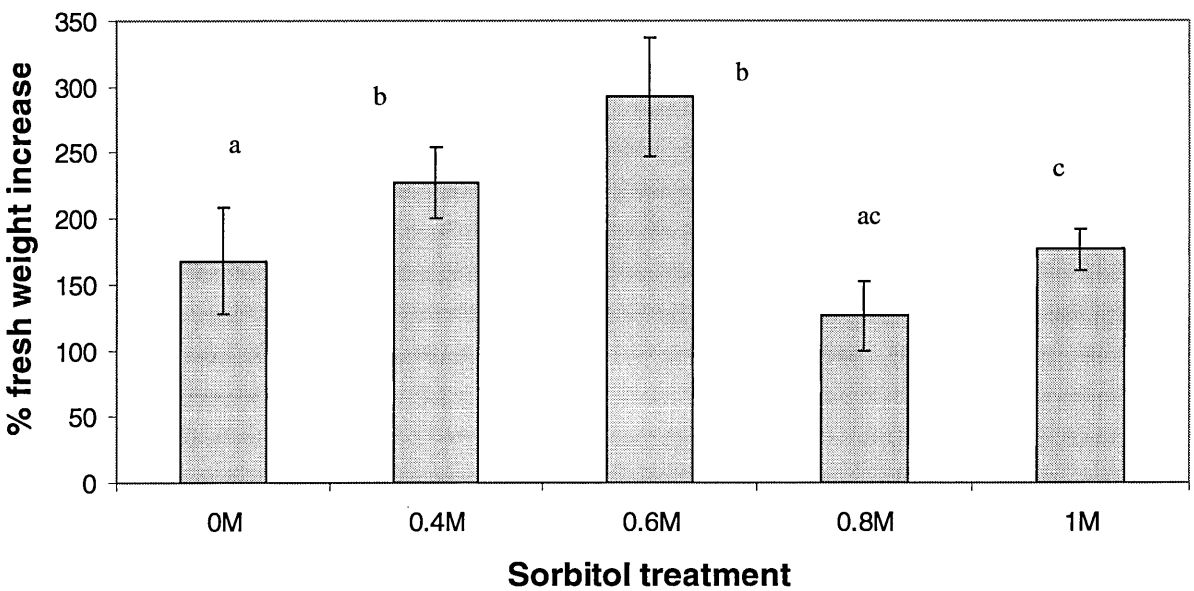


Figure 2.4 Mean % fresh weight increase of ESM following 0-1M sorbitol

Genotype E3, fresh weight at 28 days, following 48hr culture in SEMM incorporating 0-1M sorbitol concentration. Different letters denote statistically different means (± SEM) ($P<0.05$), using Fishers Pairwise comparisons.

The effect of increasing sorbitol concentration is shown in Figure 2.4, 0.4M and 0.6M sorbitol treatments showed the greatest fresh weight gain ($P<0.05$), surpassing the growth of untreated control ESM. In comparison, 0.8M sorbitol-treated cultures equalled the growth of controls. Variability (±SEM) between replicates was greatest in the control culture and was halved at concentrations of 0.8M and 1M sorbitol. A 0.4M sorbitol concentration was therefore selected for cryopreservation pre-treatments. As there was no significant difference between the 0.4 and 0.6M sorbitol treatment (Fig. 2.4) the lower concentration was selected. The optimum

time of sorbitol pre-treatment was determined (Fig 2.5) over a 10-day time course, and all ESM treatments re-grew. As the 2-6 day exposure times produced ESM recovery that surpassed the control a two-day 0.4M sorbitol culture regime was selected for all subsequent cryopreservation pre-treatment.

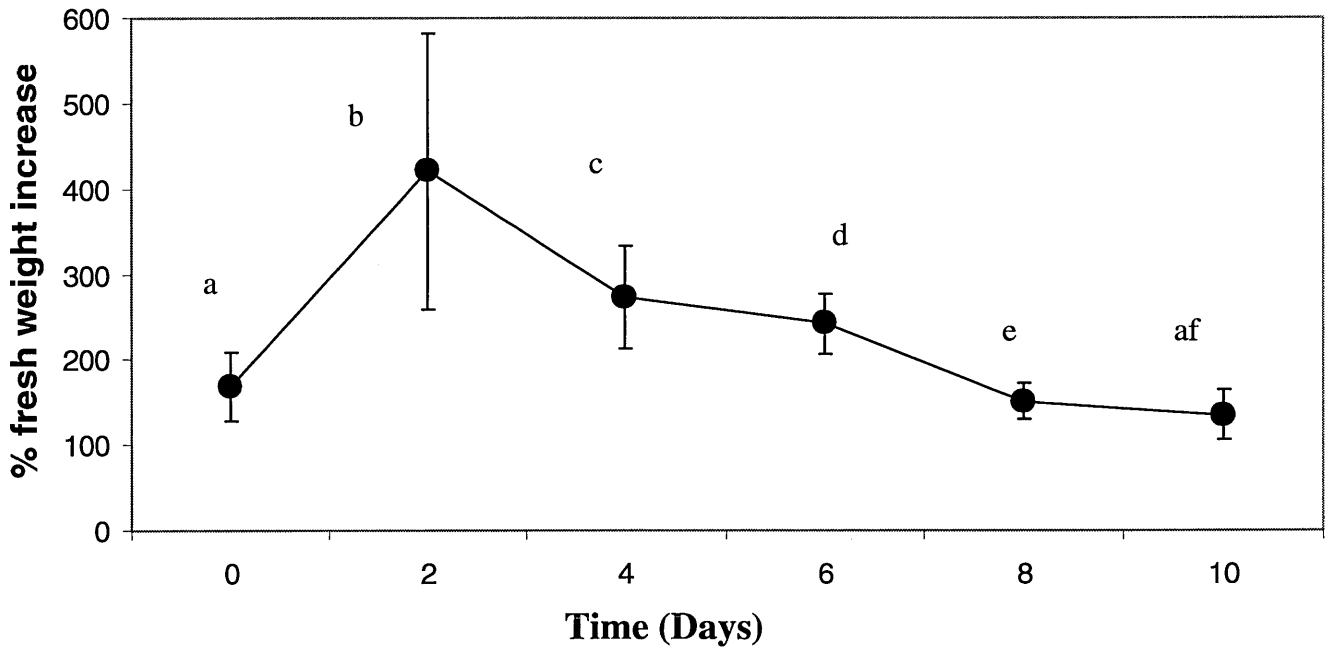


Figure 2.5 Mean % fresh weight increase of ESM following 0-10 day sorbitol treatment

Response of ESM from genotype E3 at 28 days, following culture in SEMM incorporating 0.4M sorbitol culture for 0-10 days. Different letters denote statistically different means ± SEM ($P<0.05$) using Fishers Pairwise comparisons.

2.3.2 Cryopreservation

2.3.2.1 Controlled rate cooling

Optimal cryoprotectant composition and cooling profiles were selected from the controlled rate cooling optimisation studies. Table 2.2 shows that the highest FDA viability was observed in ESM cryoprotected with 5% (v/v) DMSO, (0.95M) and cryopreserved using a cooling program that incorporated a 10 min hold at -15°C. Two cryoprotectants and two cooling profiles were tested. Cryoprotectant 1, 5% (v/v), DMSO produced an ice nucleation event at ca. -10°C and 80-100% FDA viability was observed for this treatment. Cryoprotectant 2 (Withers and King, 1980) produced an ice nucleation event at -16°C and cells recovered from this treatment were not FDA viable. Thus cooling without a hold at -15°C reduced FDA viability.

Table 2.2 Ice nucleation temperature and % FDA viability of ESM

	No hold 0- -50°C		**Hold at –15°C**	
Cryoprotectant	**Ice-nucleation (°C) ***	**FDA- % viability****	**Ice-nucleation (°C)***	**FDA- % viability****
5% DMSO control	NR	80-100%	NR	NR
WK control	NR	50-70%	NR	NR
5%DMSO -cooled	-8°C	10-20%	-10°C	10-50%
WK-cooled	-16°C	0%	-16°C	0%

Genotype A5 following cooling to -50°C no LN.

NR-Not recorded
*Ice nucleation temperatures recorded from probe printouts.
**FDA approximation determined from 5 replicates (plates).
All cultures pre-treated with 0.4 M sorbitol for 2 days and cryoprotected with either 5% (v/v) DMSO or Withers and King (WK) additive comprising 0.5M DMSO, 1M sucrose and 0.5M ethylene glycol.
Cooled ESM was pre-treated and cryoprotected as above before cooling at -0.5°C in a Controlled Rate Freezer to –50°C.
No post cooling FDA viability was observed without pre-treatment or cryoprotection.

A full appraisal of the controlled rate cooling method and experimental findings is presented in section 2.3.3.2.

2.3.2.2 Mr. Frosty™

Positive fluorescence was observed in ESM following cooling to -80°C and liquid nitrogen immersion (Table 2.3). No fresh weight increase was observed after 28 days. ESM frozen in the -20° C freezer did not show survival before or after liquid nitrogen immersion. This was a uniform response across all five preliminary genotypes selected for study.

Table 2.3 FDA viability and fresh weight response following cooling in Mr. Frosty unit

	-20°C		**-80°C**	
	% FDA viability	**% plate recovery**	**% FDA viability**	**% plate recovery**
No LN	0%	0%	20-50%	0%
LN	0%	0%	10-20%	0%

In -20°C and -80°C freezers. % FDA Viability= Fluorescein diacetate % approximation, taken from 5 replicate (plates), 4 wks after treatment. % plate recovery= % plates showing recovering ESM taken from 5 replicate (plates), 4 wks after treatment.No FDA viability observed without pre-treatment and cryoprotection

2.3.2.3 Encapsulation-dehydration

An encapsulation-dehydration protocol was developed. A 5% (w/v) Na–alginate matrix and 2 hr desiccation profile were selected during preliminary optimisation and a full cryopreservation experiment applied to the 5 genotypes selected. Four genotypes recovered following the final pre-cooling control (2hr desiccation) and one genotype (A5) showed proliferation following cryopreservation.

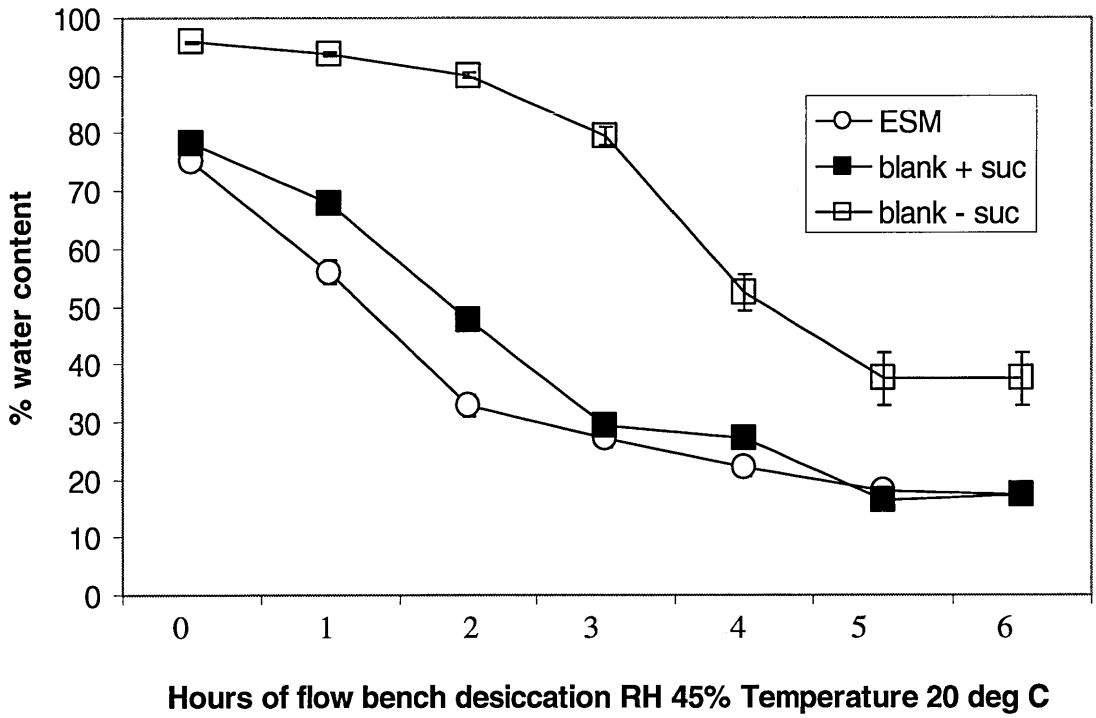


Figure 2.6 Laminar-air-flow desiccation profiles of Na-alginate beads

Changes in % water content during evaporative laminar flow bench drying as applied to encapsulated genotype A5. Data are the means ± SEM of 10 replicate beads.

ESM = Embryogenic suspensor masses encapsulated in Na-alginate beads prepared in MS medium, beads incubated in 0.75M sucrose in MS medium for 18hr.

Blank +suc= Na-alginate beads incubated in 0.75M sucrose in MS medium for 18hr

Blank-suc= Na-alginate beads with no sucrose incubation

The structure of the alginate matrix and its integrity were monitored. Beads composed of 3% (w/v) Na alginate formed a 'tail' and collapsed around the ESM during desiccation. Beads incorporating 5% (w/v) Na alginate formed symmetrical spheres and withheld integrity throughout desiccation. The water content of the alginate beads was recorded during laminar flow bench desiccation over 6 hr (Fig 2.6). Beads incorporating ESM contained less % water than blank beads. After 42 days, ESM that had been processed through all procedures up to 3hr desiccation, proliferated from the alginate bead (Fig 2.7, 2.8). Vital FDA staining indicated cell survival for all treatments, up to 4hr desiccation (Table 2.4). FDA analysis from these samples of A5 ESM indicated that longer desiccation and liquid nitrogen immersion resulted in cell destruction.

Table 2.4 ESM viability (FDA) following laminar air desiccation treatments

Treatment	**% FDA viability**
No sucrose	80-100
0.75M sucrose 18hr	80-100
*1hr desiccation	60-90
*2hr	40-50
*3hr	20-30
*4hr	5-10
*5hr	0
*6hr	0

Applied to genotype A5 after 42 days

Data are means ± SEM of 5 replicate (plates). All culture pre-treated with 0.4M sorbitol for 2 days.

*Culture pretreated with 0.75M sucrose 18hr prior to desiccation.

Based on the results from Figures 2.6, 2.7 and 2.8 a 2hr desiccation profile was incorporated into a cryopreservation protocol that was then applied to the 5 genotypes tested (Fig 2.9). Following the desiccation control, four genotypes showed 60-100% recovery in replicate beads (genotype D3 had a 100% recovery). No replicate beads showed recovery in genotype B1. Following cryopreservation, 20% of A5 replicate beads showed recovery after 28 days.

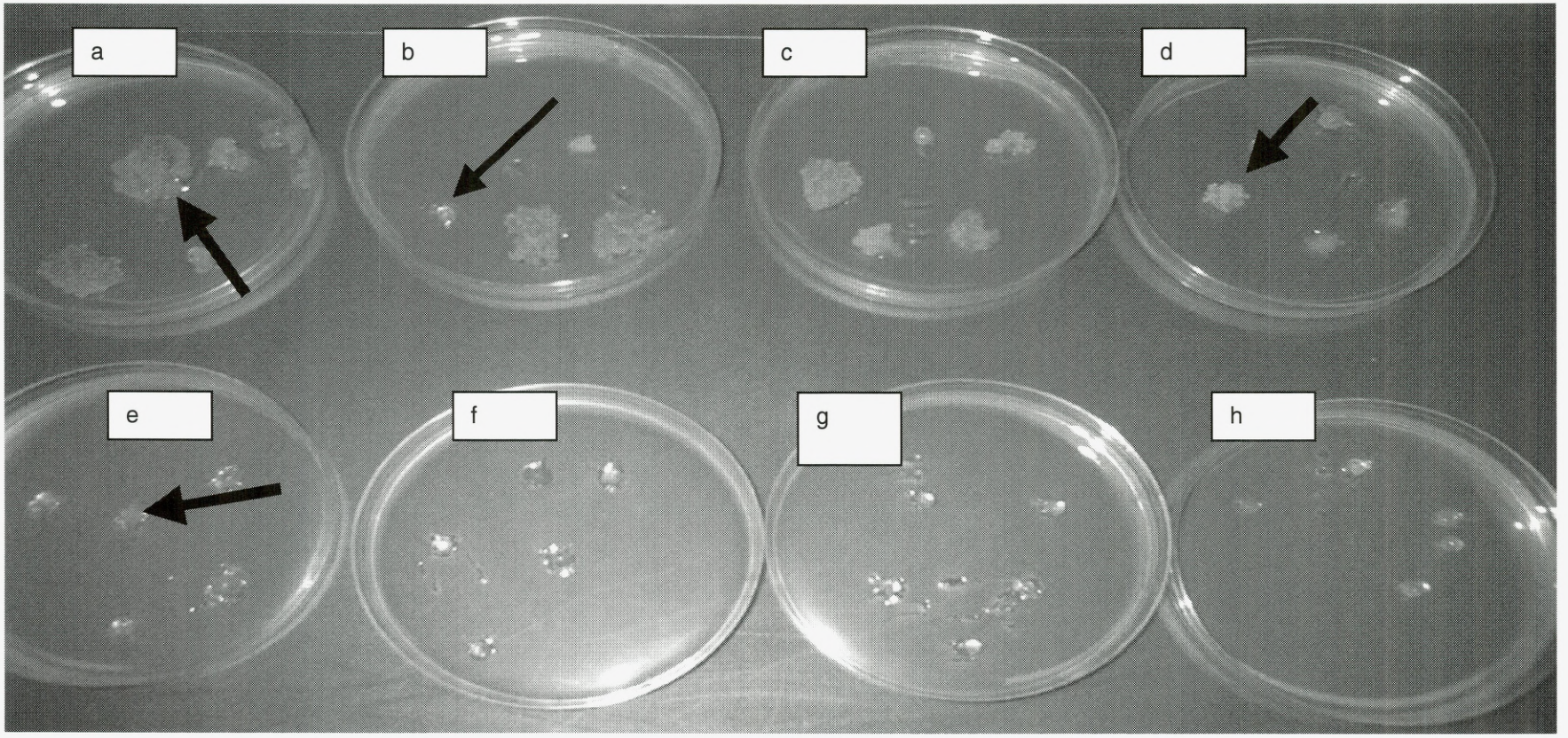


Figure 2.7 Image showing ESM recovery of genotype D3 at 42 days following laminar air-flow desiccation: a Untreated ESM arrow depicts large cell mass; b 0.75M sucrose arrows depict some beads not recovering ESM; c 1h desiccation improves recovery; d 2hr desiccation, only 1 ESM mass e 3hr desiccation, slight proliferation through one bead; f–g (4-6hr), no ESM recovery.

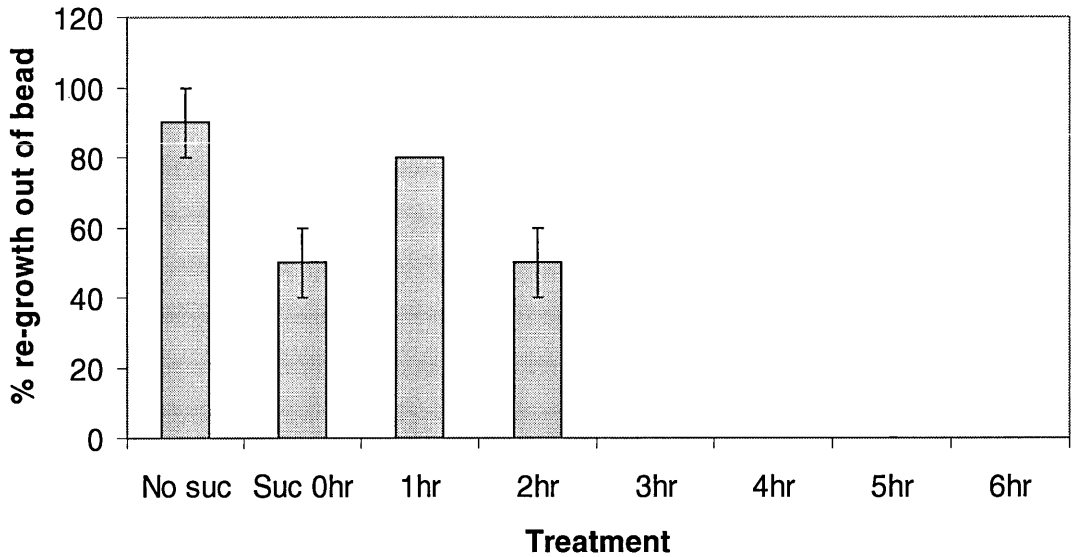


Figure 2.8 Percentage of beads showing ESM recovery following following sucrose dehydration and laminar air-flow desiccation (genotype A5)

The number of Na-alginate beads showing ESM proliferation (through the bead) after 42 days, following 0.75M sucrose dehydration and 0-6hr laminar air-flow desiccation. All ESM (genotype A5) were pre-treated with 0.4 M sorbitol for 2 days. Data are means ± SEM of 5 replicate (beads).

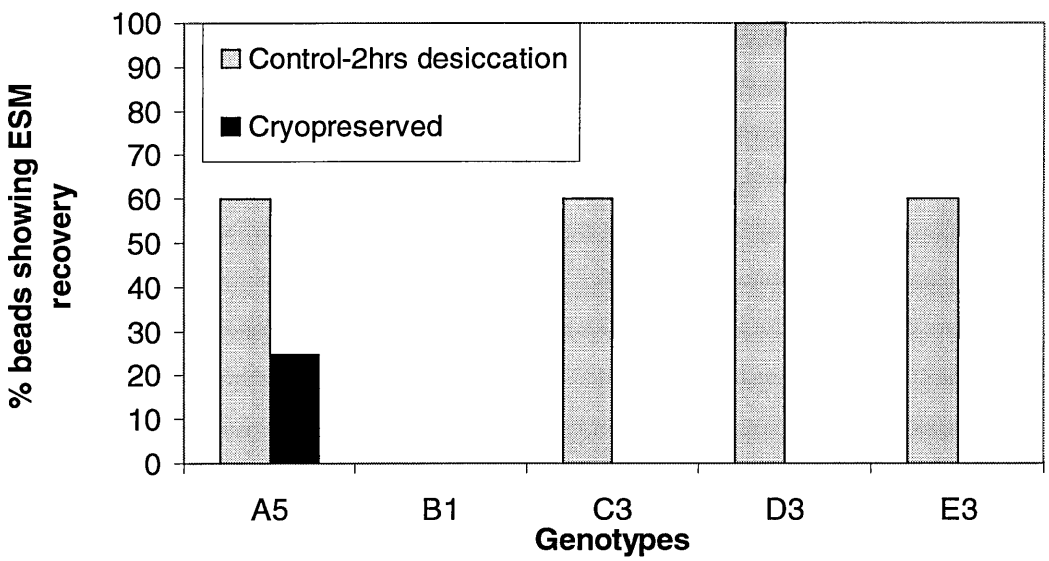


Figure 2.9 Percentage of beads showing ESM recovery following laminar air 28 days after laminar air-flow desiccation and LN treatment (genotype A-E). Data are % of beads showing ESM proliferation through the bead, following 2hr laminar air-flow desiccation or 2hr desiccation + LN treatment. Five replicates (beads) per treatment were tested in one experiment. No SEM available because experiment undertaken only once due to limited ESM availability. All ESM was pre-treated with 0.4 M sorbitol for 2 days followed by Na-alginate encapsulation and 0.75M sucrose dehydration for 18hr.

2.3.2.4 Plant Vitrification Solution 2

Toxicity and vitrification protocols were optimised and applied to the 5 ESM genotypes selected. FDA viability, % plate recovery and % fresh weight re-growth following cryoprotection and LN treatment are shown in Table 2.5. PVS2 had a major positive impact on fresh weight recovery and there was a highly variable response between replicates. In genotype A5, PVS2 treated ESM showed a greater fresh-weight increase than the untreated control (Fig 2.10).

Table 2.5 Responses of ESM genotypes following PVS2 toxicity and cryopreservation tests

Genotype	**PVS2 Cryoprotectant**			**LN**		
	FDA (%)*	**% plate recovery ****	**% F/W gain*****	**FDA (%)***	**% plate recovery ****	**% F/W gain*****
A5	30-50	100 ± 0	279-2800	0-20	0 ± 0	0
B1	30-50	60 ± 30	0-3052	0	0 ± 0	0
C3	30-50	33.3 ± 33.3	0-867	0-5	33.3 ± 33.3	0-66.7
D3	30-50	0 ± 0	0	0	0 ± 0	0
E3	30-50	0 ± 0	0	0	0 ± 0	0

* Fluorescein diacetate % approximation, taken from 5 replicate (plates), 4 wks after treatment.

**% Plates showing recovering ESM. Data are means ± SEM from 5 replicates (plates) per experiment. Experiment undertaken twice and assessed after 4 wk.

***% F/W gain = ESM fresh-weight re-growth, after 6 weeks. Data is shown as a range of % increase minimum to maximum because the response was so variable. Data taken from 5 replicates (plates).

No FDA viability observed following cryoprotection of LN without 0.4M, 2 day, sorbitol pre-treatment.

Following cryoprotection, all genotypes showed a positive FDA response and three genotypes showed fresh weight gains (Table 2.5). After cryopreservation two genotypes showed a positive FDA response and one replicate from genotype (C3) survived and recovered. After 42 days (6 weeks) PVS2-treated ESM showed a fresh weight increase that was 500% greater ($1/3^{rd}$) than the untreated control (Fig. 2.10).

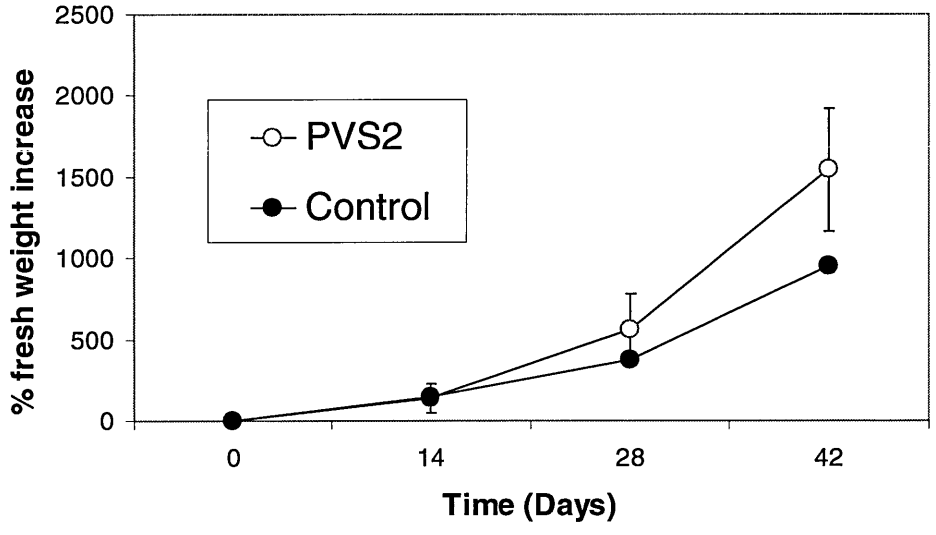


Figure 2.10 Time course of ESM recovery following PVS2 treatment ESM recovery following no treatment (control) and PVS2-treatment after 42 days, genotype A5. Data are means ± SEM from 5 replicates (plates), per experiment, each experiment was undertaken twice.

2.3.3 Protocol selection and validation

A number of criteria were specified to select the optimal protocol. Table 2.6 shows that post-cryopreservation recovery for all genotypes was observed following the controlled rate cooling protocol.

Table 2.6 Summary of cryopreservation methods and recovery

Method	Optimised pre-LN control			LN		
	FDA viability	ESM recovery	Genotype Recovery	FDA viability	ESM recovery	Genotype recovery
CRC	✓✓	✓✓	A-E	✓✓	✓✓	A-E
Mr. Frosty™	✓✓	✓✓	A-E	✓	X	NT
E-D*	✓✓	✓✓	A, C-E	NT	✓	A
PVS2	✓	✓	A-C	✓	✓	C

✓= Range of viability/recovery 0-50% X=No viability/recovery

✓✓= Range of viability/recovery 0-100% NT=Not tested

CRC= Controlled Rate Cooling; E-D=Encapsulation-dehydration; PVS2 =Plant Vitrification Solution 2

*= Based on ESM proliferation per bead. All cultures pre-treated with 0.4M sorbitol.

The controlled rate cooling protocol achieved 0-100% recoveries between replicates. After preliminary screening this method was therefore selected as the protocol of choice and applied to wider genotype screening, validation over 3 years and long-term post-cryopreservation recovery and morphogenic studies. A comparison of responsiveness between the 5 genotypes selected for preliminary study as related to sorbitol pre-treatment, cryoprotection and desiccation is shown in Table 2.7.

Table 2.7 Comparison of ESM pre-treatment responses in selected genotypes

Treatment	**Order of genotype response**	**Assessment**
Control	B1>A5>C3>D3>E3	% Fresh weight
0.4M sorbitol	A5>B1>C3>E3>D3	% Fresh weight
5% DMSO (v/v)	A5>E3>B1>C3>D3	% Fresh weight
*Loading Soln and PVS2	A5>B1>C3>D3=E3	% Plate recovery
**2hr Desiccation	D3>E3=C3=A5>B1	% Bead recovery

Order of responsiveness of different genotypes based on data derived from means $\pm$ SEM of 5-15 replicates (plates) per treatment

Note that 0.4M sorbitol 2-day pre-culture was applied before all treatments.

*Loading solution of 2M glycerol, 0.4M sucrose solution made up in liquid SEMM, pH 5.8 at 0°C was applied for 60 min, followed by Plant Vitrification Solution 2 made up in liquid SEMM, pH 5.8 at 0°C for 30 min.

**18hr 0.75M sucrose incubation followed by 2 hr laminar air flow at 20°C 45% RH.

The following section focuses on a more detailed evaluation of this cryopreservation protocol and subsequent re-growth and morphogenic studies.

2.3.3.1 Genotype screening

Nineteen of twenty-five embryogenic cell lines tested (5 families, 5 genotypes per family) achieved fresh- weight recovery following cryopreservation (Table 2.8). A General Linear Model (GLM) indicated that a significant main effect ($P<0.05$) on cryotolerance was the ESM family (Fig 2.11). The genotype was not ($P>0.05$) significant as a main effect.

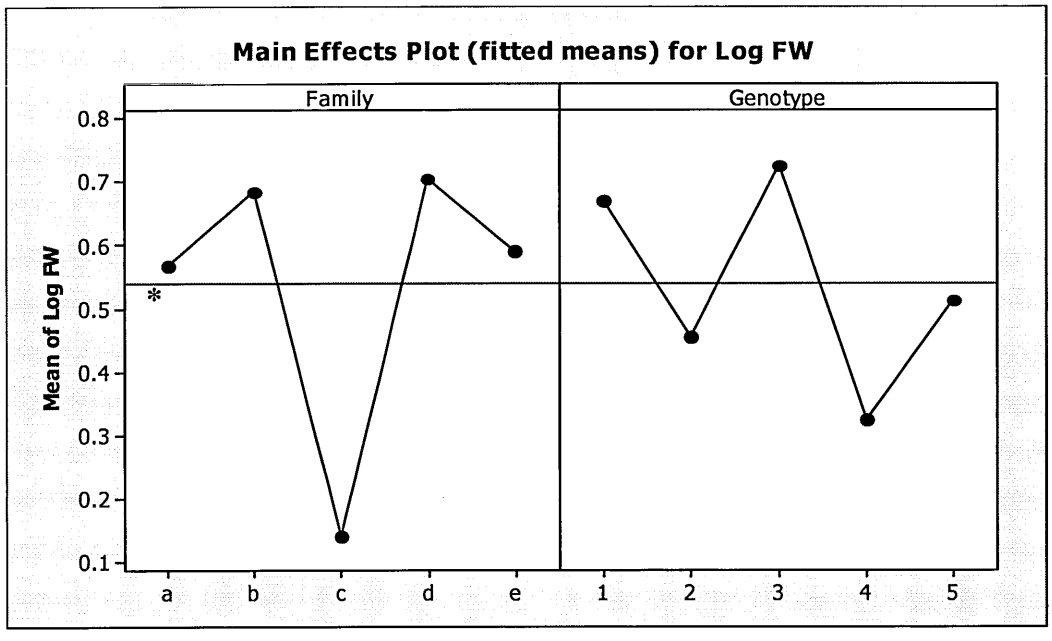
Table 2.8 Post-cryopreservation ESM recovery (genotypes A-E) following controlled rate cooling cryopreservation

Genotype	% Fresh Weight Mean ± SEM	Genotype	% Fresh Weight Mean ± SEM
A1	0 ± 0	C4	0 ± 0
A2	11.1 ± 5.4	C5	0 ± 0
A3	2.2 ± 2.2	D1	23.3 ± 13.1
A4	3.0 ± 3.0	D2	22.2 ± 13.6
A5	75.4 ± 50.3	D3	37.8 ± 8.7
B1	103.6± 83.3	D4	0 ± 0
B2	7.6 ± 2.8	D5	13.5 ± 12.9
B3	4.4 ± 2.9	E1	9.7 ± 5.6
B4	3.3 ± 2.3	E2	0 ± 0
B5	2.2 ± 1.5	E3	19.3 ± 6.91
C1	8 ± 8	E4	12.7 ± 6.0
C2	0 ± 0	E5	2.5 ± 2.5
C3	6.6 ± 6.6		

Data recorded from day 3, as % fresh weight increases over 28 days in ESM of 5 families each containing 5 genotypes. Data are means ± SEM of % fresh weight increase of 5 replicates (plates). All cultures were pre-treated with 0.4M sorbitol for 2 days and cryoprotected with 5% (v/v) DMSO.

The consistency of post-cryogenic re-growth within families can be seen in Table 2.8. The order of responsiveness of families as a group was D>B>E>A>C. The order of the highest-ranking individual genotype from each family was B1>A5>D3>E4>C1. Genotypes of family C showed a significantly ($P<0.05$) lower fresh weight increase than genotypes of other families.

Figure 2.11 The influence of family and genotype on % ESM increase following cryopreservation



Statistical package MS Minitab vs 14 model used for analysis, and General Linear Model (GLM) composed of 2 factors (genotype and family) with 5 levels/components (A-E, 1-5). Data did not meet normal distribution requirements and was logged (Log ^{10}) prior to the GLM test. Deviation from the mean mid-line indicated by * at 0.55.

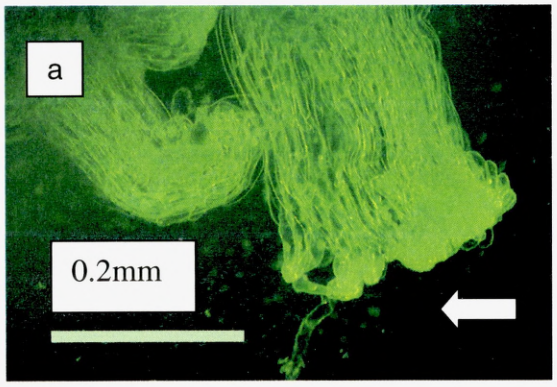
The General Linear Model (GLM) is a multi-factorial test that compares the means and standard deviations of the factors. The main effects plot shows that family C had a significantly lower ($P<0.05$) combined mean than the other families. The difference between families was significant ($P<0.05$). The main effects plot of the genotypes shows that genotype 3 from every family produced the highest mean but these differences were not significant ($P>0.05$). The differences between genotypes were not significant ($P>0.05$) at this level of analysis, in this study.

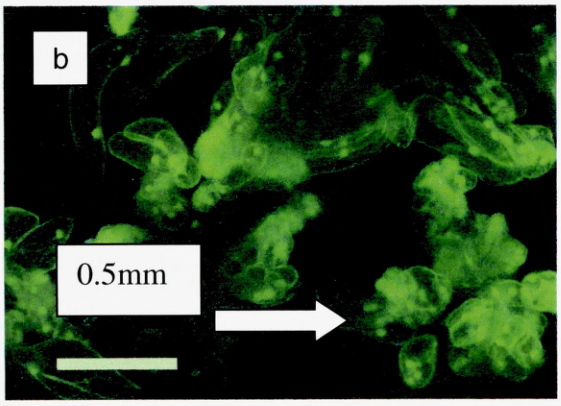
2.3.3.2 Long-term morphogenic studies

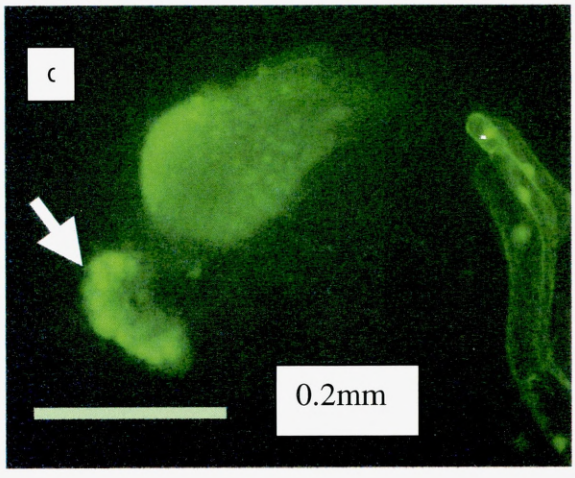
Controlled rate cooling methodology was applied to 5 genotypes over 3 years. All 5 lines of Sitka spruce embryogenic ESM survived liquid nitrogen immersion and were able to regenerate embryogenic ESM. Short/medium (3-84 day) assessments showed ESM recovery

immediately after cryopreservation in the form of FDA viability (Fig 2.12), ESM proliferation (Fig. 2.13) % plate recovery (Fig. 2.14) and % fresh weight re-growth (Fig 2.15). Visual FDA studies show recovery is morphologically heterogeneous, but that surviving meristematic tissues survive and regenerate. In experiments undertaken in Years 2 and 3, ESM re-growth was observed after cryogenic storage for all genotypes (Fig. 2.14).

Fresh weight gain of post-cryogenic first cycle sub-culture was comparable ($P>0.05$) to control cultures in one genotype (D3). Fresh weight gain in the post-cryogenic, second cycle sub-culture was comparable ($P>0.05$) to the control in 4 genotypes (Fig 2.14). In medium to long-term (2-13 months) post-cryogenic maturation, 4 lines of cryopreserved Sitka spruce ESM were also shown to proliferate as well as or significantly better than ($P=<0.05$) non-cryopreserved cultures of the same genotype and age (Table 2.9). All cryopreserved genotypes produced embryos. In two genotypes a greater number of embryos were produced per culture vessel in cryopreserved cultures than non-cryopreserved cultures (Table 2.9).







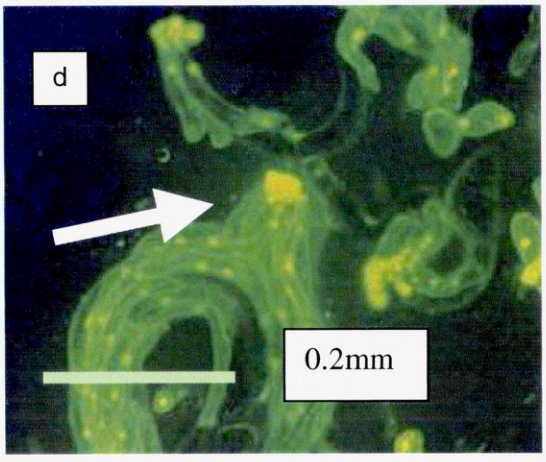


Figure 2.12. Images of post-cryopreservation morphology of *P. sitchensis* ESM cultures, visualized using FDA vital staining.

DM/RB UV fluorescence microscope used (blue/violet filter) without additional light. Images were captured using (a-c) an RGB camera and Quantimet 550 hardware with Leica UK software (d) SLR camera and film (200Exp).

a. Line A5 untreated control, Mag. X100, showing viable (see arrow) embryogenic ESM masses at mid-subculture (2 weeks) cycle.

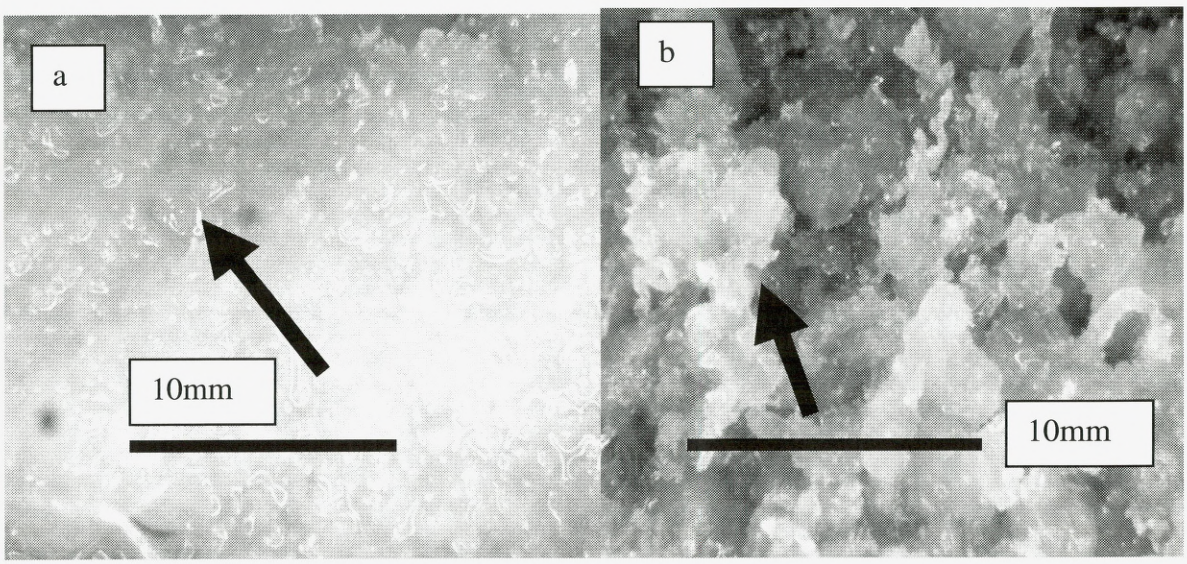
b. Line A5 pre-treated (2 days, 0.4M sorbitol) cryoprotected (5% v/v DMSO, 30 min, on ice) viable (see arrow) non-cryopreserved control, Mag. X 50.

c. Line D3 immediately after thaw ing from liquid nitrogen storage. Only the embryonal heads and suspensors (see arrows) show positive vital fluorescence for the FDA stain. Mag. X 100.

d. D3 40 days after thawing and recovery from cryogenic storage, the suspensors become detached after freezing but have re-grown (see arrow) within 40 days. Mag. X 80.

These images are representative examples of each stage of the treatment for the two most vigorously growing genotypes.

Initially, following retrieval from cryogenic storage, positive FDA was observed only in the embryonal head region of embryogenic masses; suspensor cells were disassociated and destroyed, but re-grew within 40 days of culture (Fig. 2.12d). The number of plates exhibiting ESM re-growth (Fig. 2.14) was assessed for each stage of the cryopreservation protocol. Figure 2.13 shows examples of the embryogenic status in ESM.



a) Reduced embryogenic ESM b) Embryogenic ESM

Figure 2.13. Images showing the embryogenic states of ESM.

Image (a) Arrow depicts dispersed, singular suspensor cells (b) Arrow indicates actively proliferating embryogenic clusters and embryogenic head region.

There was some variability (20-50%), through Years 1-3, in repeated experiments. In Years 1-3, all genotypes showed 100% of plate recovery at the; control, sorbitol and second post-cryopreservation subculture stage. At the next level of pre-treatment, 5% (v/v) DMSO incubation, genotypes B1, C3 and E3 recovered in all years but in genotypes A5 and D3 there was some variability. A5 re-growth was reduced by 20% in Year 1 and in D3 re-growth was reduced by 10% in Year 2. Post-LN re-growth of 100% was observed in D3 and E3 in Year 2 and 3. Two genotypes (B1 and E3) showed improved post-LN recovery rates over the three-year trial. Genotype C3 showed consistently variable recovery responses across the three years.

A two-way ANOVA of repeated measurements (which accounts for measuring over time) indicated significant differences between genotypes ($P<0.05$) for fresh weight gain (Year 3). Pre-treatment with 0.4M sorbitol for 48 h did not have a significant ($P>0.05$) effect on ESM growth as compared to untreated ESM controls and there were no differences in the responses of different genotypes to this treatment (Fig. 2.14). Cryoprotection with 5% (v/v) DMSO significantly ($P<0.05$) reduced growth after 14 days in C3 but this effect was not significant ($P>0.05$) after 28 days. In A5, 12.5% of plates did not support the recovery of ESM cultures after DMSO cryoprotection, but all other genotypes produced control-comparable recovery responses. Initial recovery after cryogenic storage was assessed at the first 42 days after thawing. The order of genotype responsiveness to cryopreservation (after $\log_{10}$ transformation) was D3>A5>B1>E3>C3. In three genotypes (B1, C3, E3) there was a significant difference ($P<0.05$) in re-growth between pre-treated and cryopreserved ESM at day 14. By day 42 only two genotypes (C3 and E3) still showed a significant difference ($P<0.05$). After the second post-cryogenic storage subculture cycle, the order of responsiveness (after $\log_{10}$ transformation) was A5>C3>D3>E3>B1. Fresh weight measurements (Fig 2.15) taken in Year 3 and at the post-cryopreservation, second subculture cycle (day 42) stage showed that throughout, all genotypes A1, C3, D3 and E3 were comparable (150-820% increase) to those of non-cryopreserved control cultures (300-950% increase) ($P<0.05$). In genotype B1, the second post-cryopreservation subculture cycle (day 42) remained depressed (200% increase) compared to the non-cryopreserved control (1145% increase). ESM showed a fresh weight increase comparable ($P<0.05$) to non-cryopreserved controls in all genotypes except B1.

ESM removed from LN in Year 2 (13 months old) and Year 3 (2-3 month old) were compared with a non-cryopreserved control ESM of an equivalent age. The mean diameter increase $\pm$ SEM after 40 days of SEABA cultures was recorded (Table 2.9) for each genotype (A-D).

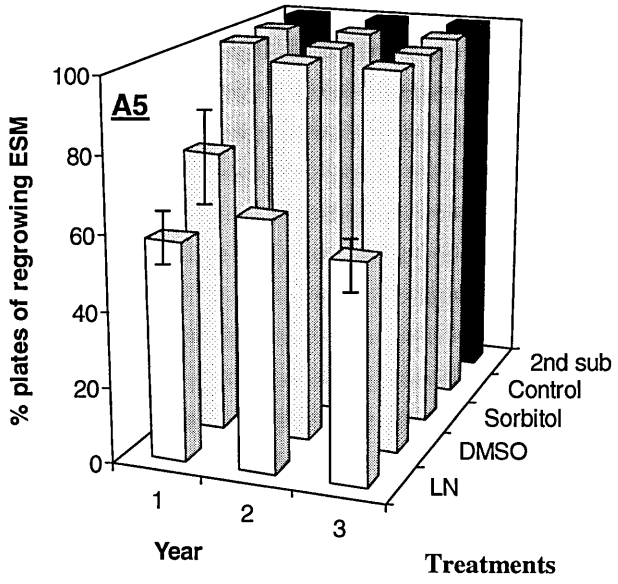
Table 2.9. Re-growth and somatic embryogenic potential of cryopreserved Sitka spruce ESM

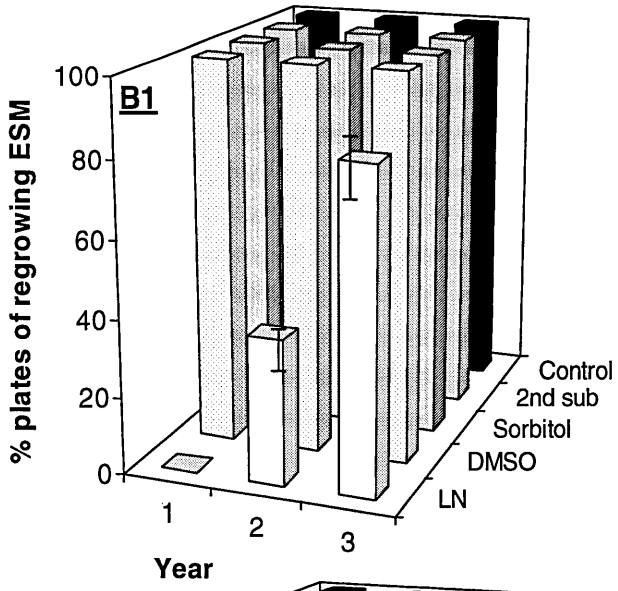
Genotype and treatment and time after cryopreservation. (10 culture tubes per treatment)	**Mean culture diameter increase (cm) after 40 days SEMM**	**Mean minimum number of embryos observed per culture tube after 10 weeks on ABA medium**		
		Stage 2	Stage 3	Stage 4
A5 – Control	8.1 ± 0.5 a	**36 ± 6**	**0**	**16.5 ± 4.5**
A5 – Cryopreserved (13 months)	6.0 ± 0.5 b	23 ± 3	0	3 ± 0.7
A5 – Cryopreserved (3months)	7 ± 0.4 ab	**38 ± 4.9**	**0**	**24.5 ± 4.5**
B1– Control	4.9 ± 0.6 a	1.7 ± 0.7	0	0.1 ± 0.1
B1 – Cryopreserved (3 months)	6.1 ± 0.5 a	1.3 ± 0.6	0	0.1 ± 0.1
C3– Control	2.9 ± 0.4 a	**21.5 ± 3.5**	**0**	**0.9 ± 0.1**
C3– Cryopreserved (3months)	3.9 ± 0.3 a	7.5 ± 2.1	0	0.4 ± 0.2
D3– Control	3.9 ± 0.3 a	1.2 ± 0.7	0	0
D3– Cryopreserved (13 months)	5.7 ± 0.4 b	**15.5 ± 2.2**	**0.2 ± 0.1**	**0.1 ± 0.1**
D3– Cryopreserved (3 months)	4.9 ± 0.2 b	0.1 ± 0.1	0	0

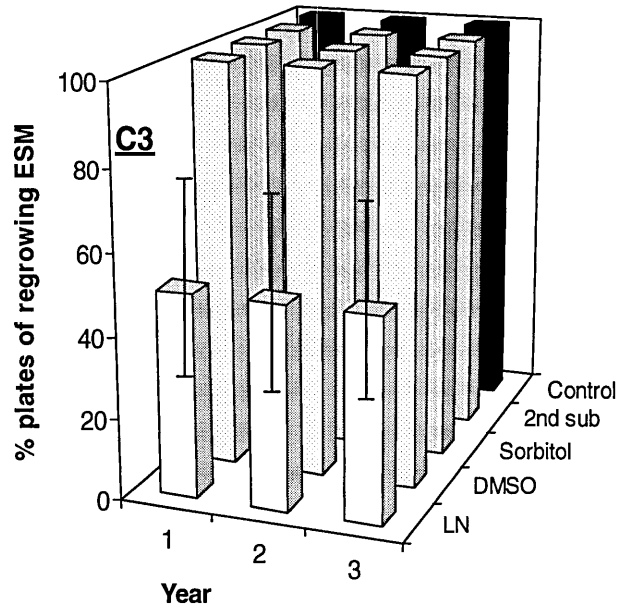
Data are means ± SEM of 5 replicates (tubes) per experiment, experiment was undertaken twice. Maturation and embryo development assessments undertaken in Year 3. Year 2 and 3 cryopreserved material stored until Year 3. Statistical analysis was used to compare non-cryopreserved and cryopreserved mean culture diameter pairs of the same genotype; means followed by same letter do not differ significantly $P>0.05$, as assessed by Students paired T-test. Bold indicates where the highest number of embryos developed in each genotype.

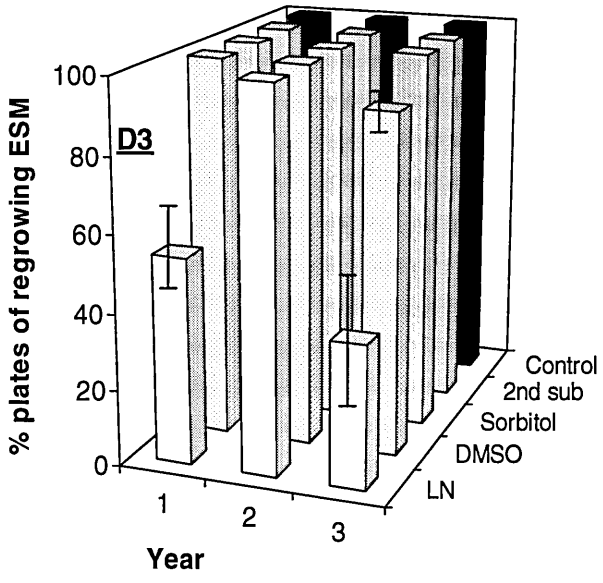
The mean culture diameter was significantly ($P<0.05$) reduced in one genotype (A5) following cryopreservation. In Year 2 cryopreserved and recovered ESM of genotype A5 proliferated significantly less ($P<0.05$) than the control. In genotype D3, cultures cryopreserved in Year 1 and 2 showed a significantly greater ($P<0.05$) culture diameter than the control. Cryopreserved and non-cryopreserved cultures produced embryos at one or more developmental stage. The order of responsiveness of the control cultures was A5<C3<B1<D3. The order of responsiveness of the cryopreserved cultures was A5<D3<C3<B1. Control B1 and D3 cell lines showed a loss of embryogenic capacity (Fig. 2.13a) at this time the cultures had been maintained *in vitro* for 5 years after initiation.

Genotype A5, ESM cryopreserved and recovered in Year 3 produced the highest number (developmental size 2, 38 ± 4.9 and size 4, 24.5 ± 4.5) of embryos per tube in the experiment. (Fig 2.13b) ESM of D3 cryopreserved and recovered in Year 2 produced 15 times more embryos per tube compared to the untreated control and to cultures cryopreserved and recovered in Year 3.









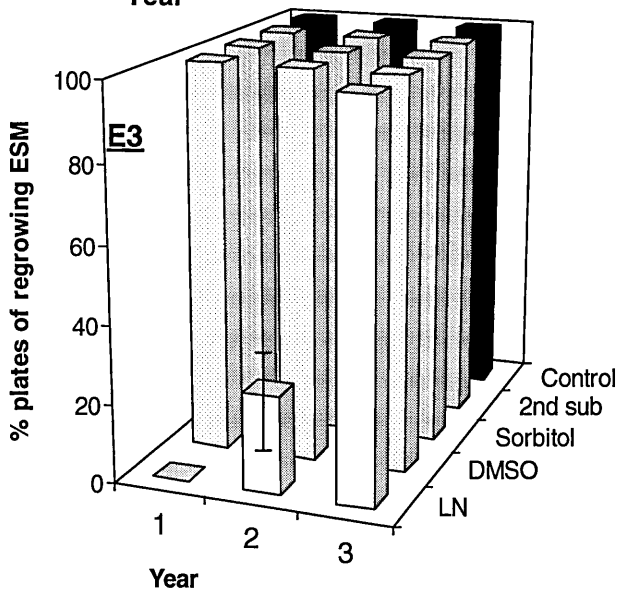


Figure 2.14. Mean % plates showing ESM proliferation (5 familes) following cryopreservation treatments. One ESM per plate per treatment showing active proliferation and fresh weight increase at 84 days. n = 5 replicates plates per experiment, duplicated experiments each year pooled to produce mean ± SEM. Repeated over Year 1, Year 2 and Year 3

Treatments

Control= culture on SEMM medium; **2nd sub**= 2nd subculture in SEMM following cryopreservation; **Sorbitol**=culture on 0.4M sorbitol in solid SEMM medium for 48hr; **DMSO**= culture on sorbitol, 5% (v/v) DMSO incubation for 30min at 0°C; **LN**= all treatment + immersion in LN for 24hr

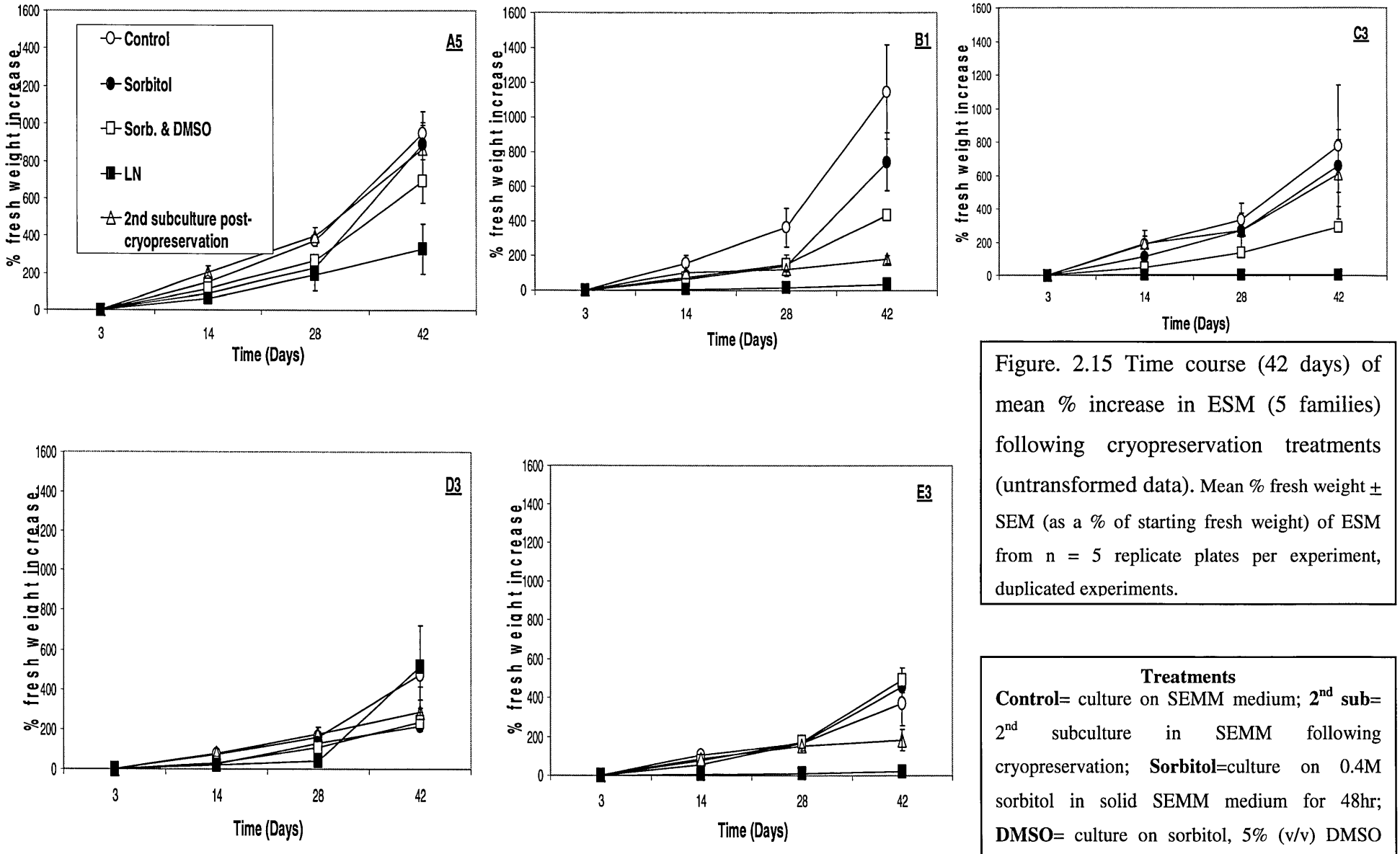


Figure. 2.15 Time course (42 days) of mean % increase in ESM (5 families) following cryopreservation treatments (untransformed data). Mean % fresh weight ± SEM (as a % of starting fresh weight) of ESM from n = 5 replicate plates per experiment, duplicated experiments.

Treatments

Control= culture on SEMM medium; **2nd sub**= 2nd subculture in SEMM following cryopreservation; **Sorbitol**=culture on 0.4M sorbitol in solid SEMM medium for 48hr; **DMSO**= culture on sorbitol, 5% (v/v) DMSO incubation for 30min at 0°C; **LN**= all treatment + immersion in liquid nitrogen for 24hr

2.4 Discussion

A controlled rate cooling protocol using a traditional colligative cryoprotection strategy and a Planer Programmable Freezer (Kryo Series 3) was developed and applied to 25 genotypes of Sitka spruce ESM cultures of which 19 recovered after cryogenic storage. A post-storage embryo maturation protocol was also concomitantly developed over the 3-year study period. The cryopreservation protocol was selected from comparatively screened, controlled rate cooling and vitrification-based (PVS2 and encapsulation-dehydration) cryopreservation methods. The controlled rate cooling method was finally selected as it produced the highest level of post-cryogenic storage recovery and, on its wider application, performed the most consistently between experimental replicates and different genotypes. Successful validation of the protocol was achieved for the five genotypes selected for preliminary study in year 1 and the subsequently more detailed study over 3 years. As a result, cryopreserved ESM was recovered and the ESM produced matured into embryos of several developmental stages.

The discussion will now consider in more detail: (1) the development and application of the selected and optimised controlled rate cooling method; (2) its applicability as compared to the other cryopreservation methods tested; (3) post-storage recovery and morphogenic studies and, (4) the application of cryopreservation to Sitka spruce germplasm conservation in forestry and tree improvement programmes.

2.4.1 Sorbitol pre-treatment optimisation

A sorbitol pre-treatment phase was found to be essential for successful cryopreservation of ESM. Pre-treatment of cells with an osmoticum, such as sorbitol, or sucrose has been shown to enhance freezing tolerance (Kartha, *et al.*, 1988, Withers and Street, 1977). Sorbitol is the pre-treatment of choice for many *Pinus* species and most *Picea* species (Cyr, 1999, Gupta and Grob, 1995) and was selected for optimisation in this applied study. It is presumed that sorbitol is not metabolised but dehydrates cells before and during the freezing process (Kartha, *et al.*, 1988). Sorbitol will therefore impart a reduction in cell size due to osmotic effects and water loss, this in turn will increase freezing resistance of the cells (Withers, 1983, Withers and Street, 1977). The non-lethal osmotic stress may also induce increased levels of ABA and late embryogenesis-abundant proteins (Charoensub, *et al.*, 1999).

Fourteen-day old ESM was selected for pre-treatment optimisation and cryopreservation. Freezing tolerance has been widely reported to be optimal towards the end of the lag and start

to mid– lag phase (Withers, 1983) and for several plant systems this corresponds with a 2-week subculture interval. A 2-day 0.4M sorbitol culture period was selected for cryopreservation pre-treatment, so confirmed, as the dehydrating treatment did not deleteriously inhibit the growth rate (Fig. 2.4, 2.5). These parameters concur with those optimised by Find, *et al.*, (1993). Interestingly, sorbitol-dehydrated cultures proliferated faster than control cultures. ESM cultured on 0.4M, 0.6M and 1M for 2 days showed statistically greater proliferation than untreated controls (Fig. 2.4). ESM on 0.4M sorbitol for 2-8 days also showed statistically greater proliferation ($P<0.05$) (Fig. 2.5).

The reason for this increased growth rate may be associated with osmotic stress-selection for younger more vigorous and meristematic (and possibly embryogenic) cells that proliferate more rapidly in the absence of older cells. The latter may be deleteriously damaged by the osmoticum and hence eradicated through stress-induced and/or natural senescence and cell death. This is also a particularly important consideration in Sitka spruce ESM, which is highly heterogeneous comprising mosaics of dedifferentiated and differentiated structures of varying cell and vacuole sizes and water contents that will respond differentially to osmotic treatments. In this study destructive quantitative examination of ESM moisture contents was not undertaken. This was primarily due to the fact that Sitka spruce cultures are slow proliferators, as compared to herbaceous plant cells. Thus, it was a technical consideration to reserve viable ESM for ongoing cryopreservation studies rather than sacrifice them for dry weight measurement at this stage. However, other researchers have undertaken studies in cryopreservation pre-treatments of asparagus embryogenic masses and determined that the % water content in calli was reduced from 90% to 70% following 0.7M sucrose incubation for 2 days (Uragami, 1993).

Another physiological response following osmotic stress reported to be associated with freezing tolerance is an increase in protein content. In *Medicago sativa* it was shown that a 0.75M sucrose culture medium produced a markedly higher level of total soluble proteins than control cultures. Additionally, the 0.75M sucrose grown cultures had a post-cryogenic viability nearly five times higher than those pre-cultured with 0.25M sucrose (Shibli *et al*, 2001).

This enhanced growth pattern does not concur with other woody species studies. Klimaszewska, *et al.*, (1992) working on *P. mariana* showed that a substantial reduction in fresh weight was observed after treatment with sorbitol at concentrations >0.4M. Indeed, during initiation somatic embryogenesis in *Prunus* sp. was inhibited by sorbitol and mannitol

(Cheong and Pooler, 2004). In this study no detrimental effects of sorbitol treatment were observed and the treatment was beneficial during maturation.

A pre-treatment of sorbitol or glycerol and not sucrose or glucose was found to be essential to survival, in several woody plant cryopreservation protocols (Touchell, *et al.*, 2002, Turner, *et al.*, 2001c). In their studies, concentrations of osmotica were adjusted to ensure a similar number of hydroxyl groups ($28.896x10^{23}$) were in the osmotica molecules. It has been hypothesised that the stereo-orientation of the hydroxyl groups of these sugar alcohols may allow for more efficient hydrogen bonding and packing around the membrane bilayer, providing increased desiccation and freeze tolerance (Turner, *et al.*, 2001a, Crowe, 1986). It is interesting that Touchell, *et al.*, (2002) found that a treatment of mannitol, a structural isomer of sorbitol (one less OH group aligned on one side), at a concentration of 0.8M produced slightly lower survival than sorbitol.

Comparative investigations into the actions of pre-treatment carbohydrates on heterogeneous ESM and homogeneous suspension cells and their relative stress responses, monitored with antioxidant and total soluble- protein levels markers, may clarify the roles of cell selection and osmotic conditioning as factors in the elevated pre-culture responses observed in this study.

2.4.2 Cryopreservation

2.4.2.1 Controlled rate cooling

Controlled rate cooling using programmable freezers has been applied successfully to several conifer species including *P. sitchensis* (Find, *et al.*, 1993), *P. mariana* (Klimaszewska, *et al.*, 1992) and *Picea abies* (Kartha, *et al.*, 1988). In this study on Sitka spruce, post-cryogenic recovery, positive FDA viability and fresh weight re-growth, was achieved through a controlled-rate cooling method, incorporating 0.4M sorbitol pre-treatment, 5% (v/v) DMSO (0.95M) as a cryoprotectant, a 0.5°C/min cooling rate, a temperature hold at –15°C (ice-nucleation observed at –10°C) and a terminal transfer temperature of –50°C. Vital staining fluorescein diacetate (FDA) assisted in protocol selection.

Two critical parameters were optimised; firstly cryoprotectant composition and secondly the cooling programme. The first cryoprotectant, 5% (v/v), DMSO selected for testing has been widely applied previously to a number of *Picea* sp. (Cyr, *et al.*, 2001). A second, multi-component cryoprotectant was chosen for comparison, Withers and King, (1980), first developed this cryoprotectant incorporating 0.5M DMSO, 0.5M glycerol and 1M sucrose, for carrot cell culture cryopreservation.

FDA examinations indicated that the peripheral location of the actively proliferating cells aided pre-treatment selection. FDA could not be used as a quantitative test due to the tissue heterogeneity. Kartha, *et al.*, (1988) and later Kristensen, *et al.*, (1994) depicted the disassociation and death of vacuolated suspensor cells of *P. sitchensis* ESM, following critical stress, that left tightly-packed embryogenic heads; this was re-affirmed in this current study.

FDA viability indicated that the Withers and King cryoprotectant showed decreased survival following cryoprotection and cooling to –40°C, compared to the 5% (v/v) DMSO cryoprotectant. The former, contained higher concentrations of penetrating (colligative) cryoprotectants in the combined form of DMSO and glycerol and the osmoticum cryoprotectant 1M sucrose. The components may therefore have exerted a greater total osmotic pressure (2M) compared to the 5% (v/v) DMSO cryoprotectant, (0.95M) used in the alternative method. Moreover, as Sitka spruce ESM is highly heterogeneous in terms of cell size and hence water content it may be more difficult to optimise a higher cryoprotectant loading, particularly for highly meristematic and younger cells. During cooling, water is drawn from the intracellular compartments to the extracellular freezing nuclei and the intracellular solute concentration will increase toxicity, osmotic stress and cell volume changes (Meryman, 1984). Cells treated with the Withers and King (1980) cryoprotectant mixture may have been assisted in their recovery by the post-thaw dilution (deplasmolysis) sucrose wash (ca.1.2M), as was applied following PVS2 treatments. The lower ice nucleation temperature (-15°C) produced by the Withers and King, cryoprotectant indicates the higher solute composition. Taking into account the above factors, the 5% (w/v) DMSO cryoprotectant was selected for further cryopreservation testing.

The incorporation of a hold or temperature plateau at -15°C in the cryopreservation programme induced an ice nucleation event in the cryovial at -8°C in the selected 5% (v/v) DMSO cryoprotectant. FDA viability indicated that this hold was essential for optimal cell survival during cooling. The "hold" supports the freeze-induced dehydration of cellular water, which is driven by the water vapour deficit, evoked by extracellular ice formation. The hold

also technically minimises the effect of localized temperature fluctuations induced by the nucleation of the media (Find, *et al.*, 1993). The optimised parameters derived from this study are similar to those reported for other conifer species (Cyr, 1999). The results pertaining to the cryopreservation testing of this protocol will be discussed in section 2.4.3.2.

2.4.2.2 Mr. Frosty™

In this study positive FDA viability was observed when cultures were cooled to -80°C and immersed in LN. Cooling to -20°C prior to immersion in LN produced no viable cells and it is likely that intracellular ice crystals were formed. Cryopreserved ESM was not recovered in this experiment. In cooling rate comparisons (Find, *et al.*, 1993) determined that a 1.0°C/min rate was sub optimal (ca. 50%) compared to a 0.5°C/min rate in fresh weight recovery. Incorporation of a temperature probe inside a cryovial contained within a Mr. Frosty™ unit during cooling may assist time optimisation. However, in the present study this method was not taken further, as the capacity to cryopreserve larger numbers of vials in the programmable freezer is more efficient for the Forestry Commission in terms of technology transfer and genebanking.

2.4.2.3 Encapsulation-dehydration

An encapsulation-dehydration protocol was developed that incorporated a 2-day 0.4M sorbitol pre-treatment, encapsulation in 5% (w/v) Na-alginate beads, 0.75M osmotic dehydration for 18hr and laminar flow bench evaporative desiccation for 2hr. There are no previous reports of applying encapsulation-dehydration cryopreservation protocols to any ESM derived from conifer species. Protocol development was based on the founding works of Fabre and Dereuddre (1990) and Niino and Sakai (1992) and through experimental optimisation. The Na-alginate matrix and the desiccation time were optimised prior to cryopreservation testing. 3% (w/v) Na-alginate beads were not spherical and were damaged easily when handled. 5% (w/v) Na-alginate beads were both sufficiently robust and sufficiently malleable to allow ESM to proliferate through to the medium during recovery. Beads containing ESM contained less % water than blank, empty beads following the same treatment. The sorbitol pre-treatment of ESM appears to influence the water status within the bead.

Encapsulated ESM showed desiccation sensitivity (Fig 2.7 and Table 2.4), and following a desiccation period of 3hr no ESM recovery was observed. Cryopreservation incorporating a

2hr desiccation [35% moisture content (MC) on a fresh weight basis] resulted in the recovery of one bead in one genotype after 42 days in culture; it is likely the MC was sufficiently high to cause critical freezing damage to the cells. No recovery was observed in cryopreserved control beads containing ESM that was not pre-treated, dehydrated or desiccated and beads appeared white when removed from LN. In embryogenic cell suspensions of grapevine (Wang, *et al.*, 2002), an optimal post-cryogenic triphenyl tetrazolium chloride viability was observed when a MC of 20.6% was acheived. A 20% MC during encapsulation-dehydration has also been recommended as a critical parameter in the cryopreservation of a number of crop species (Reed, *et al.*, 2004). In this study, an optimal pre-LN MC could not be attained without a complete loss in ESM recovery, due to the inherent desiccation sensitivity. However, it may be critical to incorporate an encapsulation-based method should the automation of embling production become necessary. An encapsulation-vitrification protocol was developed (Sakai, *et al.*, 2000) that eliminated the desiccation phase and incorporated PVS2 dehydration; this may be preferential for desiccation-sensitive, embryogenic cell cryopreservation. Desiccation sensitivity varied between genotypes. B1 ESM was unable to recover following 2 hr desiccation; A5 was able to show some recovery following cryopreservation.

2.4.2.4 Plant Vitrification Solution 2

A two-step, PVS2 method was developed incorporating a 2-day 0.4M sorbitol pre-treatment, a 2M glycerol and 0.4M sucrose, 'cryoprotectant loading,' at 0°C for 1hr and PVS2 incubation at 0°C prior to immersion in LN. No FDA viability was observed in ESM frozen without pre-treatment loading or PVS2 application. This protocol was derived from work on non-woody plant cells (Sakai, *et al.*, 1990) and through parameter optimisation of the cultures. Post-cryogenic ESM recovery was observed and progress made towards a routine vitrification method. Cryoprotectant control PVS2 produced a proliferation rate up to one third greater than that of the untreated control. To the author's knowledge there are no reports of a similar response in plant tissues. Since PVS2 cryopreservation was undertaken for this study, the first reported conifer embryogenic-culture, vitrification protocol was published for *P. mariana* (Touchell, *et al.*, 2002) using a 0.8M, 2-day sorbitol incubation followed by PVS2 incubation at 0°C for 30min. Using this method 9 of 11 embryogenic lines survived with a mean fresh weight increase from 3-67%; this report is a useful comparison for discussion.

FDA viability (demonstrated in 5 genotypes) and fresh weight recovery (1 genotype, Table 2.5) were observed following cryoprotection. ESM showed greater growth following the

application of PVS2 than the control. This has not been reported previously and as with the sorbitol pre-treatment (see section 2.4.2) may be associated with meristematic cell selection under osmotic stress. Another explanation is that the effect is due to one or more components of the loading solution and PVS2 cryoprotectant. These include glycerol (2M), sucrose (0.4M) for the loading solution, and DMSO (1.9M), glycerol (3.26M) and ethylene glycol (2.42M) for PVS2. Little work has been undertaken to examine the individual physiological affects of PVS2 components upon *in vitro* culture and the individual impacts of cryoprotectants will be discussed in the General Discussion (Chapter 7). FDA viability (2 genotypes) and fresh weight recovery (1 genotype) were observed following-vitrification. In the *P. mariana* cryopreservation detailed studies were applied to determine the optimal concentrations of compounds; this is discussed in detail in Section 2.4.1. Another difference between protocols was the ratio of ESM to PVS2 in the cryovial. In this study the ESM:PVS2 ratio was ca.1:5., in Touchell, *et al.*, (2002) study a ratio of ca.1:10 was used. Further optimisation of pre-treatment and PVS2 incubation time is therefore required to utilize this protocol in clonal forestry programmes.

2.4.3 Protocol selection and validation

It is important to select and test an optimal protocol for genotype screening, to provide a cryopreservation time-line for genebanking. Specific genotype sensitivity responses to desiccation, cryoprotectant toxicity and controlled rate cooling requires the balancing and optimisation of different factors. For example, younger more vigorous cells may achieve greater recovery with suboptimal protocols as compared to *in vitro*-aged cultures.

2.4.3.1 Genotype screening

Multi-genotype studies have been undertaken to investigate genotype and cryotolerance interactions in conifers. Noergaard, *et al.*, (1993) assessed 5 unrelated genotypes of *P. abies,* while Cyr, *et al.*, (1994) analysed 12 unrelated genotypes of *P. glauca engelmanni* complex. Both studies concur that there was a genotype (family) effect on cryo-tolerance. In contrast, (Park, *et al.*, 1998) working with 30 unrelated genotype families of *P. glauca* found no apparent genotype effect on cryo-tolerance. The results of this study, both in initial ESM survival and long-term maturation assessments, agree with Park's findings that genotype and cryo-tolerance effects may not be directly associated. This study indicates therefore that cryotolerance is associated with embryogenic capacity, which may be more predominant in some genotypes.

2.4.4 Maturation protocol development

The final stage of somatic embryogenesis within the clonal forestry system is maturation from a dedifferentiated mass to somatic embryos that are morphogenically competent and fully retain genotype characteristics capable of germinating into trees. At the time of cryopreservation a maturation method based on the work of Krogstrup (1988) and Roberts (1991) was undergoing development for selected elite embryogenic lines in the UK *P. sitchensis* breeding program. The basis of these protocols involves the application of abscisic acid (ABA) to promote synchronous embryo differentiation, development and maturation. Fully replicated field trials of mature plants have been undertaken previously for cryopreserved *Larix x eurolepsis* and *P. mariana*, (Klimaszewska, *et al.*, 1992) and *P. radiata* (Hargreaves and Smith, 1992). All plantlets from the aforementioned studies were reported to show normal phenology. In this study *P. sitchensis* genotype differences in growth and development were assessed for correlation in the mature phenotype. Useful technical information; regarding embryo production and quality across genotypes was produced.

2.4.5 Validation and long-term morphogenic studies

Short-to medium term (3-84 days post-cryopreservation), recovery was examined within 6 weeks of removal from cryostorage in five half-sibling genotype cell lines cryopreserved over a period of two years. Fresh weight proliferation rates of ESM equivalent to untreated controls were regained within the second subculture (at 84 days post-cryopreservation) in four genotypes. In long-term assessments (2-13 months post-cryopreservation) ESM cultures proliferation rates were comparable or greater than the control cultures in all four test-genotypes. Somatic embryos of developmental stages (2-4) were produced in all test-genotypes.

In short-term assessments following cryopreservation, three key factors were considered for each genotype: (1) ESM recovery after treatment; (2) proliferation rate recovery (compared to the control), and (3) length of post-treatment recovery time required. All genotypes showed post-cryogenic ESM recovery and four of the five genotypes re-established control-comparable growth rates within 84 days (Fig. 2.11). One cryo-sensitive genotype (B1) was unable to re-establish a growth rate comparable to control and showed a lag response following cryoprotectant application [5% (v/v) DMSO]. In cryopreserved cultures of *P. glauca* engelmanni complex (Cyr, *et al.*, 1994), *P. sitchensis* (Find, *et al.*, 1993) and *P. glauca* (Kartha, *et al.*, 1988) ES cultures, control comparable rates were obtained within 28 days after removal from cryogenic storage. The freely suspended cells in embryo suspensions

are possibly able to regenerate more rapidly than those contained within the heterogeneous cell mixtures typically present in ESM matrixes. Furthermore, proliferation of more homogeneous cell cultures may not be so influenced by dead or stressed cells that have been differentially affected by cryogenic treatments. Cryopreservation may thus apply a selective pressure and only those cells of suitable size and morphogenic capability tolerate the process. Vital staining indicated the distribution of cryotolerant cell clusters was heterogeneous within the ESM matrix.

Rapid regeneration was observed in genotypes A5 and D3, where dispersed regions of embryogenic clusters recovered to achieve fresh weight gains equivalent to the control within 42 days. Reproducibility was affected by the heterogeneity of the ESM. A variation of 8 to 50% in SEM values was observed across genotypes in re-growth between repeat experiments of the same year. The variation was shown to be statistically insignificant ($P>0.05$). The two-way ANOVA of repeated measures used to evaluate fresh weight increase indicated that there was no significant interaction ($P>0.05$) between genotype and pre-treatment and therefore no apparent genotype effect on cryo tolerance of ESM.

In long-term assessments, all four test genotypes showed active proliferation and produced somatic embryos at development stages 2–4. There was a genotype difference in culture size and somatic embryo production. Genotype A5 showed the greatest culture diameter increase and produced Stage 4 embryos; the level of development reported as optimal for successful transfer and germination (Gupta and Grob, 1995). All four genotypes were able to proliferate and produce embryos after cryopreservation. In two genotypes (A5 and D3) culture proliferation and embryo maturation was improved in the post-cryogenic cultures. The association between culture surface area expansion and somatic embryo production may be important. It has been observed in newly maturing embryos that development irregularities can occur when embryos come in to contact with each other (Svobodova, *et al.*, 1999). Asynchronous development and differences in somatic embryo morphology were apparent between genotypes as was observed in a number of other species including barley hybrids (Croke and Cassells, 1997), spring barley (Chernobrovkina, *et al.*, 2004) and *P. abies* (Bozhkov and Von Arnold, 1998). Morphological and biochemical studies of *Picea* somatic embryo development are assisting in the continual development and improvement of maturation protocols (Thorpe, 2000).

Improved embryo uniformity and production may be associated with 0.4M sorbitol treatment. The benefits of osmoticum application in a maturation protocol have been highlighted and carbohydrate supply is known to be an important factor (Stasolla, *et al.*, 2002, Thorpe, 2000).

In artificial seedlings of Siberian ginseng sucrose was included in the encapsulation matrix doubling the germination rate of somatic embryos (Jung, *et al.*, 2004). *In vivo* zygotic embryos undergo a maturation process with programmed desiccation, *in vitro* the application of osmotica may function to remote maturation. A better understanding of these stress factors and their role in modifying endogenous hormones levels associated with dormancy and hence physiological status is required to assure asynchronous development during maturation and has important implications for morphogenically competent cells recovering from cryopreservation..

This study incorporated some assessment of culture health and cryotolerance after prolonged *in vitro* storage. The short-term treatment response of *in vitro*-aged ESM cultures was monitored over 3 years. In control, sorbitol and 2nd subculture ESM there was no difference in recovery from Years 1–3 across genotypes. Cryoprotectant toxicity (0-20%) was observed in two genotypes in Years 1 and 3. Cryogenic impacts varied across genotypes and from Year 1 to 3. Three patterns were observed over three years; (1) consistent recovery (C3), (2) increased recovery in cryogenic-sensitive genotypes (E3 & B1) as the operator performed procedures more efficiently (Reed *et al*, 2004) and (3) decreased recovery as embryogenic capacity and culture heath deteriorated through prolonged time in culture (B1, D3 and A5).

The long-term effects of a 3-year *in vitro* culture period in two control cultures (B1 and D3) included a loss of embryogenic capacity. Cryotolerance of Year 2 and 3 cultures, (as indicated by proliferation during maturation) was apparently unaffected by culture age ($P<0.05$). Cryotolerance of Year 2 and 3 cultures, in respect to embryo production, was associated with embryogenic capacity which in some genotypes decreased with age. In D3 embryogenic capacity was lost in Year 3 of experiments and so both the control and post-cryogenic ESM produced that year showed reduced (Stage 2) or nil (Stage 4) embryo maturation. ESM cryopreserved in Year 2, at a time when embryogenic capacity was still present, produced a minimum mean of 15 Stage 2 embryos per tube. In contrast, improved embryo maturation in A5 was observed in Year 3, this might be associated with improved protocol efficiency.

In this study, embryogenic capacity was lost through prolonged *in vitro* culture (5 years) in some genotypes. Gupta and Grob, (1995) discussed the observed differences between research in *P. abies* regarding the stability and embryogenic potential maintenance, which varied from 6 months to 1 year. Physiological and biochemical investigations may reveal the causal factors of this loss. In *P. abies* it has been reported that developmental and maturation capabilities are influenced by the loss and gain of paternal characteristics during prolonged *in*

vitro storage (Niskanen, *et al.*, 2004). In rice suspension cultures embryogenic cells survive cryopreservation better than non-embryogenic cells (Lynch, *et al.*, 1995). A physiological explanation is that there is a requirement for tight metabolic coupling and that this may not be the case in older aged cultures which may be more disposed to cryogenic stress (Benson, *et al.*, 1992). This in turn, may reduce post-thaw injury associated with changes in glycolytic and respiratory intermediates. Rice embryogenic cells are also known to have a higher antioxidant status than non-embryogenic rice cells (Benson, *et al.*, 1992). *In vitro* aged cultures generate larger stressed cells that are likely to have lost their meristematic characteristics making them more difficult to cryopreserve due to their water status. In shoot tips of *Solanum tuberosum* post-cryogenic survival was significantly reduced in long-term (3 year) tissue cultured plants when compared to short-term (6-8 weeks) maintenance (Harding, *et al.*, 1991).

2.4.6 Applications in forestry

Somatic embryogenesis is an integral component of *in vitro* conifer improvement programs. Cryopreservation is essential for the storage and accelerated deployment of germplasm derived from selected genotypes and allows the evolution and use of tree populations arising from concomitant long-term field trials (see Fig.1.2 in Introduction). *Picea* clonal forestry cryopreservation programs have been in progress since the 1990s; *P. mariana* (Adams, *et al.*, 1994), *P. englmanni* (Complex) (Cyr, *et al.*,1994) including, *P. abies* (Noergaard, *et al.*, 1993), *P. sitchensis* (Find, *et al.*,1993) and *P. glauca* (Park, *et al.*, 1993).

In this study a controlled rate cooling protocol using a Programmable Freezer was applied to ESM of five Sitka spruce families each with five genotypes. Several genotypes showed post-cryogenic recovery (19 of these 25 genotypes). The protocol will now be applied by the UK Forestry Commission's Northern Research Station to 50 families over the next three years, each family containing 10 genotypes. A recommendation of cryopreservation within six months of initiation has been proposed. Mr. Frosty and PVS2–based protocols may be developed over time. Post-cryogenic germination, phenotype and genetic stability will be evaluated as the programme progresses.

This study is the second conifer investigation to explore the possible effects that the period of post-LN culture multiplication has on commercial deployment. Somaclonal variation was detected in *P. glauca* embryogenic cultures 2 and 12 months following recovery from cryostorage but this did not affect the corresponding regenerated trees (De Verno, *et al.*, 1999). In this *P. sitchensis* study 2-3 month and 12-13 month post-cryopreservation cultures,

from different cryopreservation experiments, were compared for culture health and embryo capacity (Table 2.9). Increased time in storage produced cultures comparable to the control, and the influencing factor appeared to be the health and embryogenic capacity of the pre-cryopreservation. Further investigations using cultures from the same cryopreservation experiment may clarify the critical time frame of post-cryopreservation multiplication time.

2.5 Conclusions

A cryopreservation protocol, using a programmable freezer, was successfully developed for the cryopreservation of *P. sitchensis* embryogenic suspensor masses (ESM). The protocol was applied to 25 genotypes, of which 19 showed survival, and was validated over three years. Several cooling protocols were tested. The programmable freezer method produced the greatest recovery followed by 'Mr. Frosty' (Nalgene®); Plant Vitrification Solution 2; and the least recovery was observed using an encapsulation-dehydration method. These results suggest that the ESM is more sensitive to laminar air desiccation than to the toxicity incurred during vitrification.

The programmable freezer method was applied to 5 unrelated families each with 5 half-sibling genotypes, 19 of 25 genotypes recovered from cryopreservation (Table 2.8). Statistical analysis determined that there was a significant ($P<0.05$) difference in cryotolerance between families and not between genotypes (Fig. 2.11). The method was validated over three years, using five selected genotypes, and post-cryopreservation recovery was observed every year in three genotypes (Fig. 2.14). Post-cryopreservation fresh weight re-growth was comparable to control growth within 84 days of re-warming (Fig. 2.15).

In maturation testing, all genotypes produced embryos following cryopreservation; cryopreserved cultures often showed improved health and embryo production as compared to untreated control cultures (Table 2.9). In this study cryotolerance was associated with embryogenic capacity, which is genotype and age dependent. It is known that embryogenic capacity decreases over time so it is highly recommended that cultures are cryopreserved soon after their initiation and specifically it is recommended that cryopreservation is undertaken within 6 months of culture initiation. In Chapter 3 a cryopreservation protocol for the somatic embryos (matured ESM) has been developed and the influencing factors investigated.

Chapter 3 CRYOPRESERVATION OF SOMATIC EMBRYOS

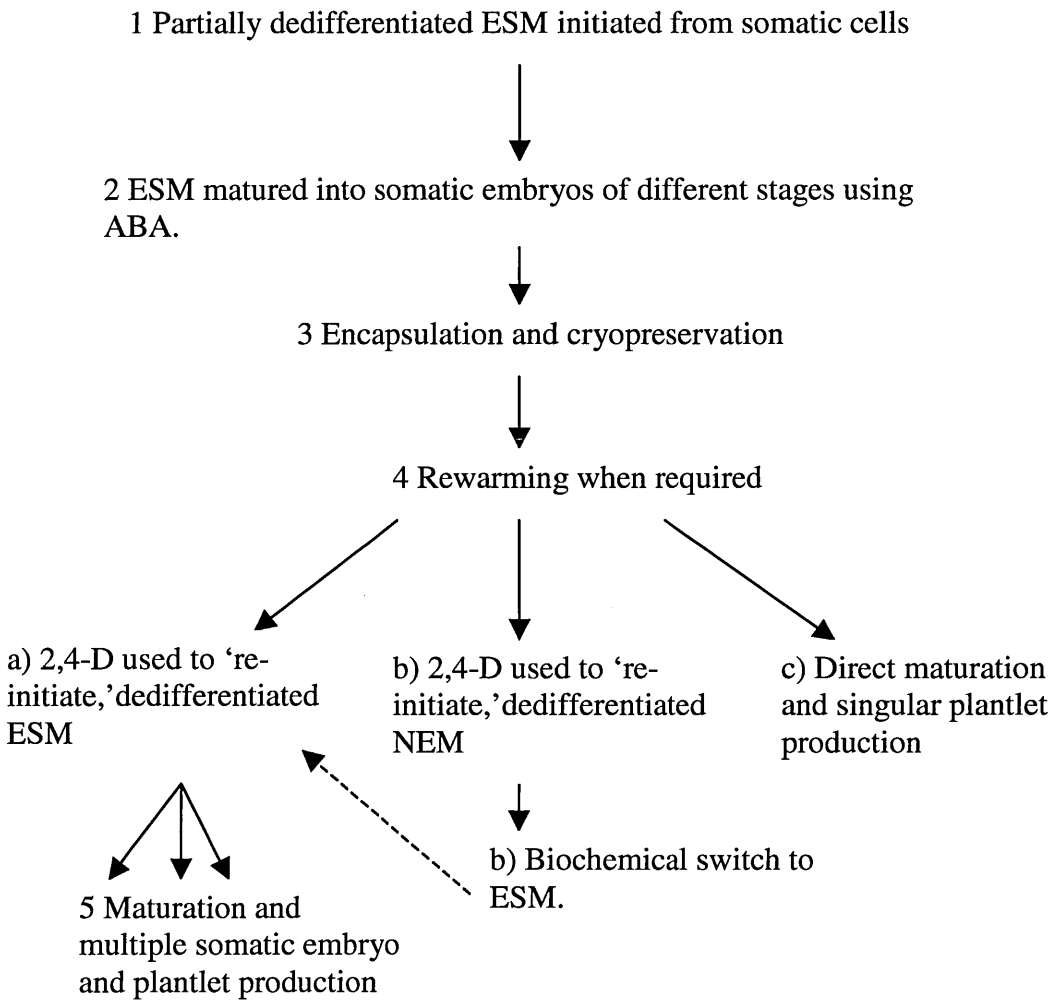
3.1 Introduction

The objective of this chapter is to develop a robust cryopreservation protocol for matured somatic embryos. These cryopreserved somatic embryos will form the starter culture for the initiation of embryogenic suspensor masses that will be used to multiply elite clonal germplasm prior to maturation and plantlet production. This objective will be achieved through pre-treatment and cryoprotectant optimisation and the development of encapsulation and non-encapsulation based protocols. Further aims will include assessing the effects of genotype and *in vitro* storage on cryotolerance and re-initiation of cultures after cryogenic storage.

3.1.1 *In vitro* somatic embryo development in conifers

Somatic embryogenic tissue can be obtained from a single immature or mature zygotic embryo extracted from conifer cone seed (Gupta and Grob, 1995). Somatic embryogenic tissue mimics zygotic embryogenic tissue, resulting in somatic embryos that can mature, germinate and form plantlets. This tissue culture method is widely used for clonal plantlet production and offers advantages over nursery methods in forestry by shortening the clone generation time for field-testing. The first report of somatic embryogenic capacity in conifers was from immature zygotic embryos of *P. abies* (Hakman, *et al.*, 1987). A number of detailed *in vitro* studies investigating the biochemistry, development (Stasolla, *et al.*, 2002) and desiccation tolerance (Percy, *et al.*, 2001) of embryogenic tissues and somatic embryos have since been undertaken.

Figure 3.1 Somatic embryo cryopreservation and plantlet production system



ESM= Embryogenic suspensor masses
2,4-D= 2,4-dichlorophenoxyacetic acid
ABA= abscisic acid

3.1.2 Commercial application and cryopreservation

Cryopreservation is required to maintain germplasm in long-term storage while, the often lengthy, clonal field-testing is undertaken. A proposed system of somatic embryo cryopreservation and plantlet production is shown in Figure 3.1. The size and facilitated handling of matured somatic embryos, compared to friable ESM, may be beneficial for encapsulation-dehydration and PVS2 vitrification cryopreservation protocols (Fig, 3.1.3). Following cryopreservation, the matured somatic embryos already possessing pre-formed shoot and root meristems, may, with the correct culture, differentiate directly into seedlings without any lag phase (Tessereau, *et al.*, 1994), (Fig. 3.1.4c). They may also revert to a dedifferentiated embryogenic suspensor mass (Fig. 3.1.4a); hence a single embryo may be clonally propagated to potentially produce thousands of the selected clonal genotypes, ready for maturation and delivery to the forester.

Detailed characterisation of *P. glauca* (Hakman and Arnold, 1985) and *P. sitchensis* (Kristensen, *et al.*, 1994) (Chapter 2, Fig. 2.3) somatic embryo development has been undertaken. Research in *P. glauca* somatic embryos (Stasolla, *et al.*, 2002) suggests that increasing lipids and protein content and cotyledonary development (increased histodifferentiation) is initiated as the embryo matures into a cotyledonary state. These factors are likely to affect water status, vacuole size and therefore cryopreservation tolerance.

This study will investigate pre-cotyledonary globular size 2 embryos and mature cotyledonary size 3 embryos (Chapter 2, Fig. 2.3). These somatic embryo developmental stages were selected in order to determine the effects of cryopreservation on different stages and to make comparisons with similar *Picea spp.* investigations, (Bomal and Tremblay, 2000).

Encapsulation-dehydration cryopreservation protocols have been used to cryopreserve somatic embryos from a number of species including coffee (Hatanaka, *et al.*, 1994), cocoa (Fang, *et al.*, 2004) and black iris (Shibli, 2000). Cryopreservation protocols using encapsulation-dehydration and encapsulation-vitrification have been applied to somatic embryos of woody plant species including olive (Shibli and Al-Juboory, 2000).

Conifer somatic embryos of *P. glauca* and *P. glauca* x engelmanni complex (Percy, *et al.*, 2001) and in *P. mariana* and *P. glauca* (Bomal and Tremblay, 2000) have been cryopreserved without encapsulation using desiccation over salt solutions. Although the germination of encapsulated somatic embryos of *P. glauca* and *P. mariana* has been investigated (Lulsdorf,

et al., 1993), cryopreservation of conifer somatic embryos via encapsulation-dehydration has not yet been reported. Encapsulation-dehydration of embryos involves encapsulation in a protective Na-alginate bead followed by sucrose–dehydration and laminar air-flow desiccation to an optimum moisture content. At this water content the intercellular cell compartments are sufficiently vitreous with concentrated solutes to circumvent ice formation and somatic embryo cells are expected to regenerate following cryogenic storage.

A PVS2 based encapsulation-vitrification protocol was successfully applied to olive somatic embryos (Shibli and Al-Juboory, 2000). This protocol involves encapsulating embryos in a protective Na-alginate bead, followed by dehydration and osmoprotection in a vitrification solution, such as PVS2, usually containing a mixture of highly concentrated cryoprotectants. Optimum exposure to the PVS2 solution ensures that ice nucleation is circumvented and a stable amorphous glass is produced upon rapid cooling to -196°C. Vitrification, using PVS2, of naked somatic embryos has been applied to the monocotyledon *Macropidia fuliginosa* (Turner, *et al.*, 2000). There are no reports of vitrification or encapsulation-vitrification application to conifer somatic embryos.

An encapsulation-based protocol is desirable for forestry somatic embryo cryopreservation because: (1) it reduces injury through pre-treatment handling; (2) offers the possibility of automation and (3) may be incorporated in 'synthetic seed' production pathways (Gray and Purohit, 1991). Following encapsulation, cryopreservation and re-warming embryos can be directly matured into emblings and greenhouse plantlets. Another developmental pathway is to place somatic embryos on 2,4,-D allowing some cells to revert to dedifferentiated embryogenic suspensor masses (ESM), a process termed 're-initiation' or 'secondary initiation'. This dedifferentiated mass can then be used to generate tens of thousands of identical embryos for maturation and plantlet production. It may prove a preferential pathway for 'bulking–up', for plantlet production in forestry programmes. This has been achieved for non-encapsulated *Picea* sp. (Bomal and Tremblay, 2000), where embryogenic tissue was regenerated and subsequently developed into mature somatic embryos at rates comparable to control donor cultures.

Reports suggest that a proportion of non-embryogenic mass (NEM) or callus is often produced during re-initiation and during direct plantlet development (Shibli, 2000, Shibli and Al-Juboory, 2000, Tessereau, *et al.*, 1994). Although this non-embryogenic mass cannot be used directly to produce mature embryos, little is known about the relationship between embryogenic and non-embryogenic masses and if certain treatments (such as sorbitol pre-treatment) influence the developmental pathway. An investigation on plant growth regulators,

carbon sources and iron influence on secondary somatic embryogenesis in transgenic cherry rootstock suggested that 1mg thidiazuron (TDZ) with 2% (w/v) sucrose reverted morphogenic callus to non-morphogenic callus (Gutierrez and Rugini, 2004). Clearly, further investigations are required to determine if: (1) the non-embryogenic masses can be induced to become embryogenic and yield somatic embryos and (2) how genetically stable this production route is. Dedifferentiation can induce more genetic changes than regeneration through a differentiated route and may be associated with somaclonal variation problems (Harding, *et al.*, 1996). In this study the percentage of ESM and NEM regeneration will be monitored.

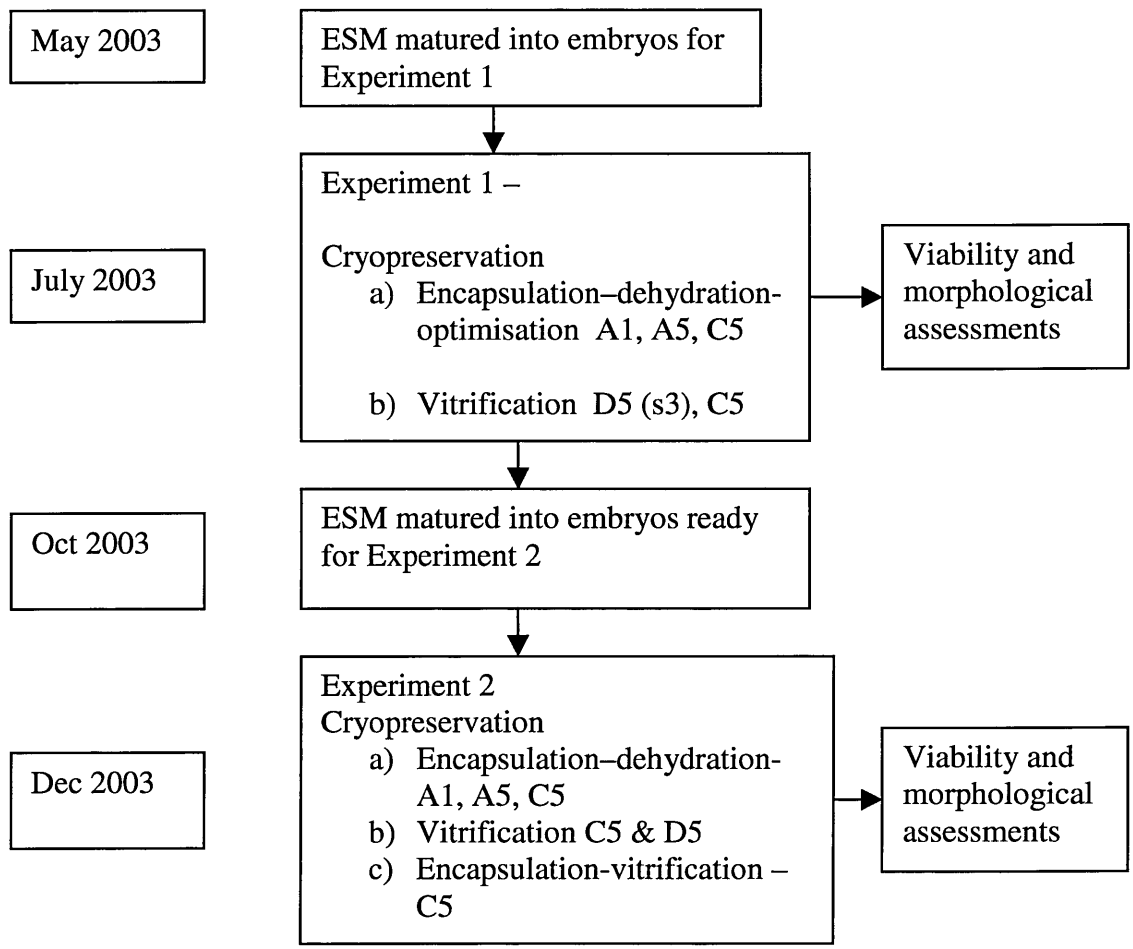
In this investigation, somatic embryos of two developmental stages, from four genotypes will be cryopreserved using encapsulation-dehydration and encapsulation-vitrification protocols. As the cryopreservation protocol has never been applied to *P. sitchensis* previously, optimisation will be undertaken to determine laminar air-flow desiccation requirements, and the post- encapsulation, desiccation effects over time (4 months). Following encapsulation-dehydration and encapsulation-vitrification the encapsulated embryos will be placed on medium containing 2,4-D and induce NEM and ESM.

3.2 Materials and Methods

3.2.1 Experimental design

Somatic embryos of *P. sitchensis* cannot be maintained in a particular or synchronised developmental state and therefore careful planning to mature ESM is critical to produce embryos for cryopreservation testing. Figure 3.2 shows the experimental strategy required to mature somatic embryos ready for cryopreservation and assessment. Five families (A-E) each containing five genotypes (1-5) of ESM were matured, but not all were able to produce embryos. Genotypes producing the most embryos were selected for testing. C5 produced the most embryos of all genotypes and was incorporated in every cryopreservation method. To avoid depleting the core NRS stock the same genotype was not always available for selection in experiment 1 and 2. Vital staining (TTC) and morphological assessments were undertaken during the 3 months after testing to determine viability and development after cryogenic storage.

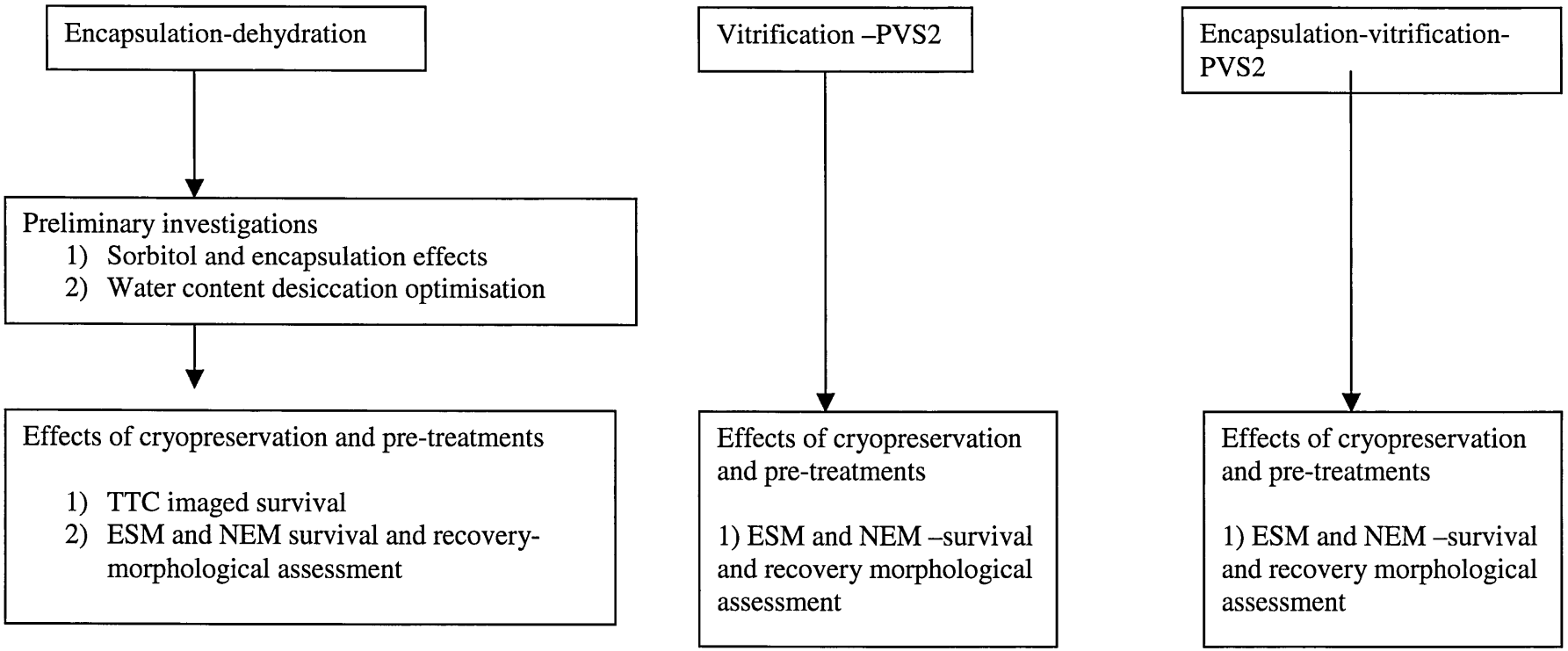
Figure 3.2 Experimental design and timeline for the cryopreservation of somatic embryos



3.2.2 Somatic embryo development and maturation

Embryogenic suspensor mass (ESM) establishment and all details of Somatic Embryogenic Medium (SEM) are described in Chapter 2, Materials and Methods. Embryo maturation studies were undertaken at Northern Research Station, Roslin using genotypes selected on the following criteria: representation of each genetic group, significance for Forestry Commission breeding programmes and culture vigour.

Figure 3.3 Experimental plan of cryopreservation protocol investigations



TTC= Tetrazolium Triphenyl Chloride staining; ESM =Embryogenic suspensor mass; NEM =Non-Embryogenic Mass; PVS2=Plant Vitrification Solution 2

Cultures were maintained for 6 weeks on SEMM before transfer to Somatic Embryogenic Activated Charcoal Medium, SEAC, (10g/l, 25mls per plate, pH 5.7) without plant growth regulators for 1 week (John, *et al.*, 1995). The cultures were then transferred to Somatic Embryogenic Abscisic Acid Medium, SEABA (John, *et al.*, 1995) containing 25mg/l abscisic acid (ABA) for 6-10 weeks.

Embryos were transferred onto hormone-free SEMM, incubated in the dark at 20 $\pm$ 1°C, ready for cryopreservation pre-treatments. Genotypes A1, A5, C5 and D5 were selected because they consistently produced embryos. Embryos of development stage 2-globular (0.5 x 0.5mm) and 3 heart-shaped to torpedo (0.5-1.0 x 0.5-1.0mm) (Fig. 2.3, see Chapter 2) were selected for cryopreservation testing. After selection the embryos were placed on SEIM and 0.4M sorbitol for 48hr (in the dark at 20 $\pm$ 1°C).

3.2.3 Re-initiation of ESM and NEM

Somatic embryo re-initiation was undertaken on Somatic Embryogenic Initiation Medium (SEIM) containing 0.43mg/l kinetin, 11.5mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.45mg/l 6-benzylaminopurine (BAP) (John, *et al.*, 1995). The morphogenic responses of the somatic embryos were monitored as: (a) no developmental change; (b) reversion to ESM, and (c) production of NEM.

3.2.4 Cryopreservation protocols

A plan of the three cryopreservation protocols tested is shown in Fig. 3.3. All somatic embryos were treated with 48hr of 0.4M sorbitol in solid SEMM, and their responses investigated in preliminary investigations. The effects of alginate encapsulation and laminar air-flow desiccation are also investigated. Tetrazolium triphenyl chloride (TTC) staining upon whole embryos as a viability assessment is applied to post encapsulation-dehydration embryos. The main assessment of somatic embryo post-cryopreservation survival and morphological capability is the development of ESM and NEM.

3.2.4.1 Encapsulation-dehydration

Stage 2 and 3 embryos were selected and pre-treated as described in 3.2.2. Residual ESM was removed from pre-treated individual embryos that were then encapsulated (using 3ml sterile-single Pasteur-plastic pipettes, Fisherbrand) to produce beads (ca. 4mm diameter/50µl volume). The encapsulation procedure derived from Benson, (1993) and Fabre and Dereuddre (1990) incorporated 3% (w/v) sodium alginate (alginic acid, low viscosity SIGMA A-21580) made up in liquid SEIM without $CaCl_2$ and dispensed into 100 mM $CaCl_2$ solution made up in liquid SEIM. The beads were left to polymerise for 20 min, and encapsulated embryos blotted on filter paper and then osmotically dehydrated in 0.75M sucrose solution (made up in liquid SEIM) and placed on a shaker (bench top, 120 rev/min CERTOMAT ®) for 18hr (in the dark 20 ± 1°C).

Encapsulated somatic embryos were cooled and re-warmed as described in 3.2.5. Following re-warming the beads were re-hydrated in liquid SEIM for 20min. Somatic embryos were recovered as detailed in 3.2.6.

(a) Preliminary investigations sorbitol pre-culture, alginate encapsulation and laminar air-flow optimisation

Sorbitol pre-culture and alginate encapsulation

A detailed time-course of the effect of sorbitol and encapsulation on embryo re-initiation was undertaken. The % of size 2 and 3 embryos developing embryogenic suspensor masses (ESM) and non-embryogenic masses (NEM) following no treatment, 0.4M sorbitol and Na-alginate encapsulation was monitored once a month for 4 months. Ten replicate embryos per treatment per experiment were tested, and the experiment was undertaken twice. Data was plotted as the mean ± SEM (between experiments), genotype C5 was assessed in these studies.

Laminar-air-flow optimisation

The optimal desiccation time was determined by TTC viability and moisture content analysis. The moisture content of 10 empty (no tissue) beads was determined by weighing each bead immediately after sucrose incubation (T0) and then after each hour (Tx) of desiccation (up to 6hr). Beads were first blotted on filter paper and then desiccated (on an open bottom plate of 90mm diameter, sterile plastic Petri dishes, Sterilin™) in a laminar flow bench (45% RH and 20°C). After 6 hr the beads were placed on separate glass Petri dishes (45mm diam.) and were placed in a 105°C oven for 12 hr (Tdry). Each bead was reweighed. The following formula was used to calculate the water content for each bead.

((Tx-Tdry)/Tx)*100

The mean ± SEM were determined.

Where:

Tx= Fresh weight of bead at each desiccation time (0-6hr)

Tdry= Weight of bead following drying in an oven at 105°C for 12hr.

(b) Cryopreservation survival and re-initiation examinations

TTC survival following 14 days

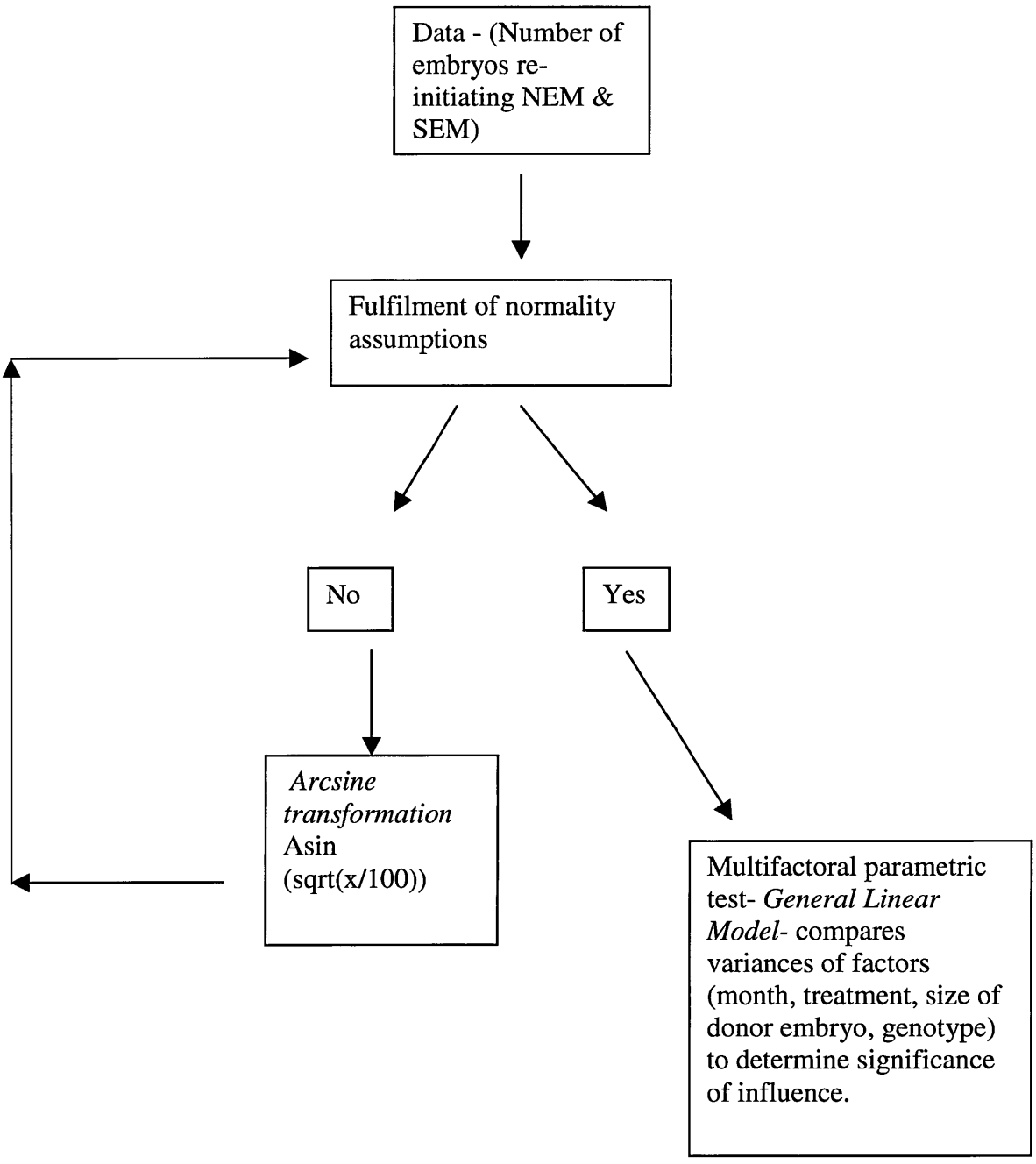
Stage 2 and 3 embryos were tested; from genotypes A1, A5 and C5 in July 2003 for TTC viability (3.2.6.1) at day 14, following: (1) control, (2) sorbitol pre-treatment and encapsulation, (3) desiccation and, (4) cooling in LN. The experiment consisted of two batches (five embryos per batch) and the number of embryos showing 50% or more red formazan staining after overnight incubation was recorded as survival. The mean ± SEM between blocks was determined. After re-hydration: 10 embryos (2 batches of 5) per treatment/genotype were tested for TTC viability; 10 embryos (2 batches of 5) per treatment/genotype were transferred to solid SEMM (5 per tube) and were tested for TTC viability (see 3.2.5.1) after 14 days, and 20 embryos per treatment/genotype were transferred to solid SEIM.

ESM and NEM proliferation from cryopreserved somatic embryos

Stage 2 and 3 embryos were tested; from genotypes A1, A5 and C5 in July 2003 and December 2003. Morphological indicators were used (see 3.2.6.2) to indicate ESM and NEM re-initiation. Ten replicate somatic embryos were tested per treatment, per genotype, per experiment and each experiment was undertaken twice. Data from month 4 was assessed. Data shows mean ± SEM between experiments. Statistical analysis, was used to determine the level of significance of each of the following factors: month of testing, developmental state, genotype and treatment upon the re-initiation of NEM. Multi-factorial factor analyses are shown in Fig. 3.4.

It was not known if the colour of treated somatic embryos and proliferating ESM or NEM, was an important characteristic of recovery. In other *in vitro* plant explants a brown colour has been associated with culture degradation and phenolic oxidation (Svobodova, *et al.*, 1999). The colour (white, beige or brown) of treated and untreated embryos was compared with the % of embryos showing ESM and NEM production and a correlation test (Spearmans Rank, rs) was used to determine the statistical relationship between ESM/NEM recovery and colour.

Figure 3.4 Statistical Analysis Flowchart



Data processes in statistical analysis to determine significance of factors affecting Non-Embryogenic Mass (NEM) and embryogenic suspensor masses (ESM), re-initiation from somatic embryos.

3.2.4.2 Plant Vitrification Solution 2 (PVS2)

Stage 2 and 3 embryos were selected and pre-treated as described in 3.2.2. A 2M glycerol, 0.4M sucrose loading solution and PVS2 solution [30% (w/v) glycerol, 15% (w/v) DMSO, 15% ethylene glycol (section 2.2.5.4)] prepared with liquid SEIM instead of SEMM. Five embryos were placed in a 2ml cryovial (Simport™, self standing) and 2M glycerol, 0.4M sucrose solution was added to a final volume of 1.8ml at 0°C for 60min. Embryos were transferred to freshly prepared pre-cooled (4°C for 1 hr), PVS2 solution on ice for 30 min. Cooling and re-warming was as described in 3.2.5. Embryos were transferred to sterile Wilson sieves (100µm) pore size and rinsed with 1.2M sucrose made up in liquid SEIM at pH 5.8. Embryos were assessed for ESM and NEM recovery using the morphological indicators described in 3.2.6.2, following no treatment, PVS2 application and cryopreservation. Stage 2 and 3 embryos were tested; from genotypes A1 and C5 in July 2003 and from genotype C5 in December 2003. Five to ten replicate embryos were tested per treatment, per genotype, per experiment and each experiment undertaken twice.

3.2.4.3 Encapsulation-Vitrification (PVS2)

Stage 2 and 3 embryos were selected and pre-treated. A protocol was developed based on 2M glycerol, 0.4M sucrose loading solution and PVS2 solution (Sakai, *et al.*, 2000, Shibli and Al-Juboory, 2000). Pre-treated somatic embryos were encapsulated in 2% (w/v) Na-alginate beads containing 0.4M sucrose. Encapsulated somatic embryos were incubated in 2M glycerol and 0.4M sucrose for 1 hr on a rotary shaker at + 20°C followed by PVS2 incubation for 3 hr at 0 °C. Embryos were transferred to 2 ml cryovials (Simport™, self standing) and re-suspended in 1.8ml of fresh PVS2 (5 encapsulated embryos per cryovial). Cooling and re-warming was undertaken as described in 3.2.5. Embryos were transferred to sterile Wilson sieves (100µm) pore size and rinsed with 1.2M sucrose made up in liquid SEIM at pH 5.8.

Embryos were recovered and assessed using morphological indicators described in 3.2.6.2, following encapsulation, PVS2 application and cryopreservation. Stage 2 and 3 embryos were tested; from genotype C5 in December 2003. Five replicate somatic embryos were tested per treatment, per genotype, per experiment and each experiment undertaken twice.

3.2.5 Cooling and re-warming

Vials were placed on cryo-canes with sleeves and immersed in LN for a minimum of 24hr. Cryovials were removed from liquid nitrogen and allowed to re-warm on a polystyrene float

on a 40°C water bath for 2-3 min. Cryovials were surface sterilised (Hibitane®) and sterile liquid SEIM was added to the cryovial for 20 min to re-hydrate the embryos.

3.2.6 Recovery and assessments

Embryos were placed on solid SEIM in soda glass tubes (1 per tube) and sealed with clingfilm. Viability and re-initiation assessments were undertaken.

Viability testing using Triphenyl Tetrazolium Chloride (TTC) vital staining

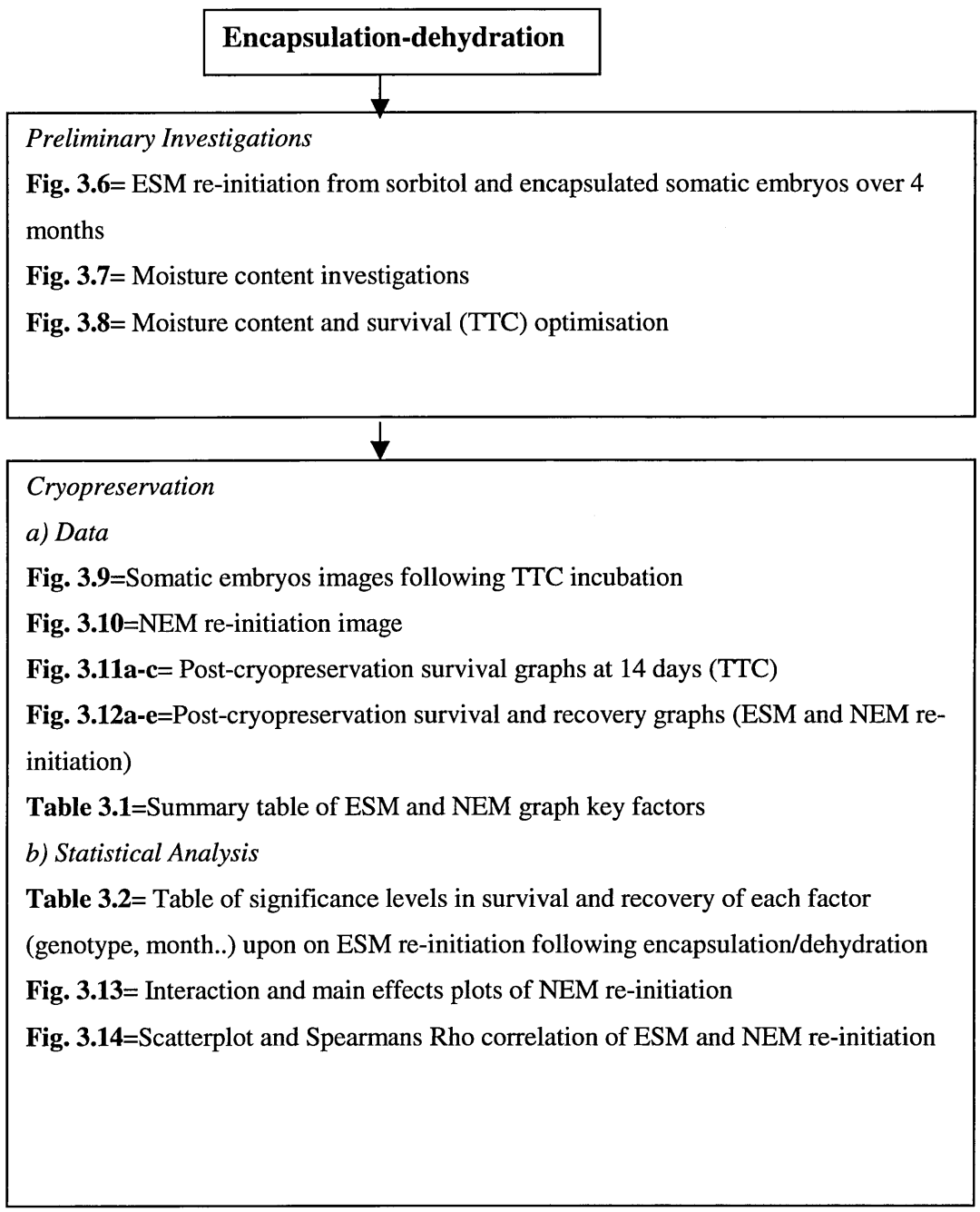
Somatic embryos were maintained at UAD in the dark at 20 ± 1°C for re-growth monitoring. To enable the optimum desiccation time with optimum viability assessments TTC (Benson, 1993, Steponkus and Lanphear, 1967) testing was used. Stock reagents 0.05M Na_2HPO_4 and 0.5M KH_2PO_4 were buffered to pH 7.4. 0.6% (w/v) of TTC was added to the stock reagents + 0.05% (v/v) Tween 80. Somatic embryos were placed in 20ml plastic centrifuge tubes (5 per tube) and 3ml of TTC reagent added and infiltrated under vacuum for 10-20 min. The tubes were then incubated at 28°C overnight. Somatic embryos were assessed under a binocular microscope. Dehydrogenase activity within viable cells reduces TTC to a red formazan product.

ESM and NEM re-initiation morphological indications

Somatic embryos were transported to Northern Research Station, Roslin for re-growth monitoring. The cultures were maintained in the dark at 20 ± 1°C. The morphological descriptors used to assess recovery were colour (white, beige or brown) and if the encapsulated embryo was unchanged, swollen, producing ESM or NEM. The number of embryos from each batch of 5 to 10 showing recovery in each morphological descriptor category was compared with the replicate batch to produce a mean ± SEM. Embryos were assessed once a month, for 4 months; they were not transferred to new medium during these assessments.

3.3 Results

Figure 3.5 Guide to results for cryopreservation protocol investigations



Vitrification

a) Data

Fig.3.15a-d=NEM and ESM re-initiation graphs

Table 3.3= Summary table of ESM and NEM graph key factors

b) Statistical Analysis

Table 3.4- Table of significance levels in survival and recovery of each factor (genotype, month) upon on ESM re-initiation following PVS2 vitrification.

Fig. 3.16= Main effects plot of ESM re-initiation

Fig. 3.17= Main effects plot of NEM re-initiation

Encapsulation-vitrification

No results obtained following contamination

3.3.1 Encapsulation –dehydration

An encapsulation-dehydration protocol was developed and successfully applied to somatic embryos of three genotypes (A1, A5 and C5) at two developmental sizes [immature (2) and mature (3)]. The protocol comprised 2 days pre-growth in 0.4M sorbitol in SEIM; incubation in 0.75M sucrose in liquid SEIM for 18hr, 4hr laminar air-flow desiccation and LN immersion. The procedure was optimised using: osmotic dehydration studies (Fig. 3.6), moisture content assessments (Fig. 3.7), and viability staining using Tetrazolium Triphenyl Chloride (Fig 3.8 and 3.9). Following a full cryopreservation trial, experimental results were assessed in two ways: firstly, through the immediate survival of embryos (TTC staining), and secondly through growth as re-initiation and proliferation of embryogenic suspensor masses, ESM, and non-embryogenic masses, NEM. Following LN immersion genotypes A5 and A1 showed post LN survival after 14 days, all genotypes re-initiated NEM and C5 re-initiated ESM (Table 3.1).

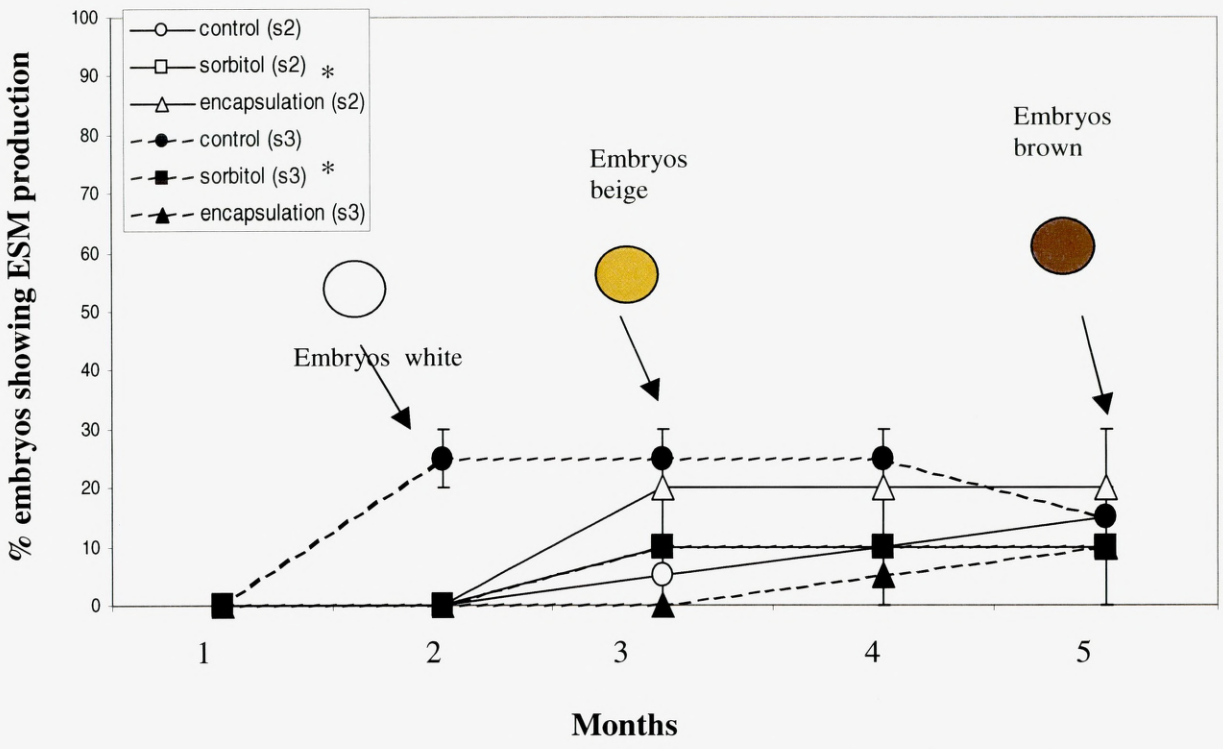


Figure 3.6 The number (%) of embryos showing ESM re-initiation following treatment at monthly sampling intervals for 5 months

* Sorbitol s2 and s3 both marked by ■

Genotype C5, size s2=size 2 and s3 =size 3 embryos. ESM = Embryogenic suspensor masses

Treatments were undertaken on 19th July 2003. Naked somatic embryos were untreated (cultured on SEIM only); pre-treated with 0.4M sorbitol on SEIM and then re-cultured on SEIM alone; encapsulated in 3% (w/v) Na-alginate, and maintained in beads during assessments. Each experiment comprised of ten replicates beads and each experiment was duplicated and pooled to produce % mean ± SEM.

In preliminary experiments, the effects of sorbitol and encapsulation were tested over 5 months from day 1, (Fig. 3.6); 0.4 M sorbitol and Na–alginate encapsulation did not reduce re-initiation; indeed encapsulated immature embryos and sorbitol pre-treated mature embryos showed a greater ESM recovery than the untreated controls. Without Na-alginate encapsulation, embryos became shrivelled and were unrecoverable. Figure 3.6 shows the colour change, across the somatic embryos, from white to brown over 4mth. This was observed in all treatments and a statistical test of correlation (Spearmans Rho) showed there was no significant relationship (P=0.155) between the colour of the embryo and the re-initiation of either ESM or NEM.

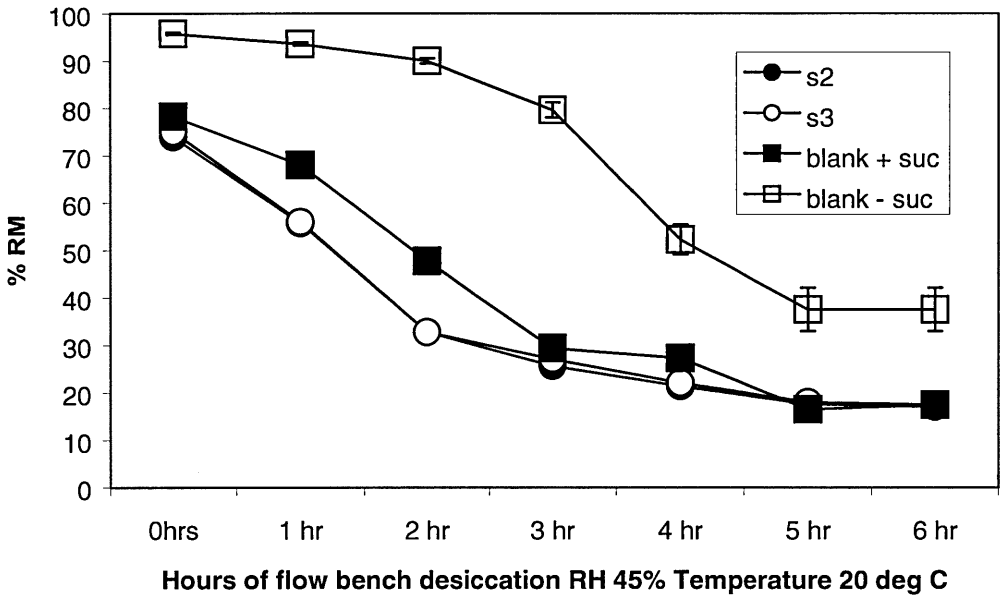


Figure 3.7-Moisture contents of blank Na-alginate/SEIM beads and A5 size 2 and 3 embryos. Blank – suc = no additional sucrose; Blank + 0.75M sucrose in liquid SEIM for 18hr. S2 (size 2) and S3 (size 3) embryos in 0.75M sucrose. Ten replicate beads ± SEM.

% RM = % Relative Moisture content= ((Tx-Tdry)/Tx) X 100 where Tx=fresh weight of bead after desiccation and Tdry= fresh weight of bead after 12hr 105°C oven drying.

Preliminary investigations also optimised moisture content through laminar air-flow desiccation to maximise relative moisture loss but minimise viability loss (Figs 3.7 and 3.8). The water content of encapsulated size 2 and 3 embryos following all pre-treatments at (RH 45%, temperature 20°C and 4hr laminar air-desiccation) was ca. 20% (Fig. 3.7). TTC testing indicated that the embryos showed some desiccation sensitivity as viability was reduced after 4-5hr of laminar air-flow desiccation (Fig. 3.8). There was variability in embryos viability (10-60% between repeated experiments). TTC imaging showed that regeneration was mostly observed from the mitotically active basal embryo area in the stage 3 embryos (Fig. 3.9d). This is also the area where ESM was actively proliferating (Fig. 3.10). In the torpedo shaped embryos, cell integrity was observed at the top cotlyledonary region (Fig 3.9f). The optimum desiccation time was 4hr with a moisture content of ca.20%.

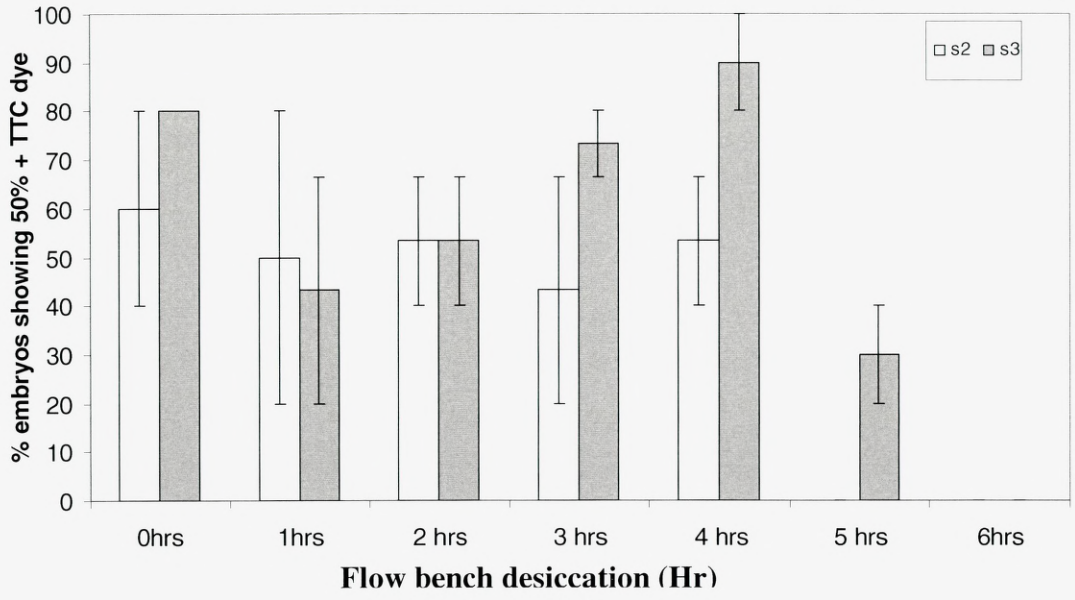


Figure 3.8. Survival plot of encapsulated somatic embryos following laminar air-flow flow desiccation

Genotypes A1 and C5, desiccation 0-6hr.

n=10 replicate beads $\pm$ SEM.

S2 = size 2 somatic embryos and S3= size 3 somatic embryos

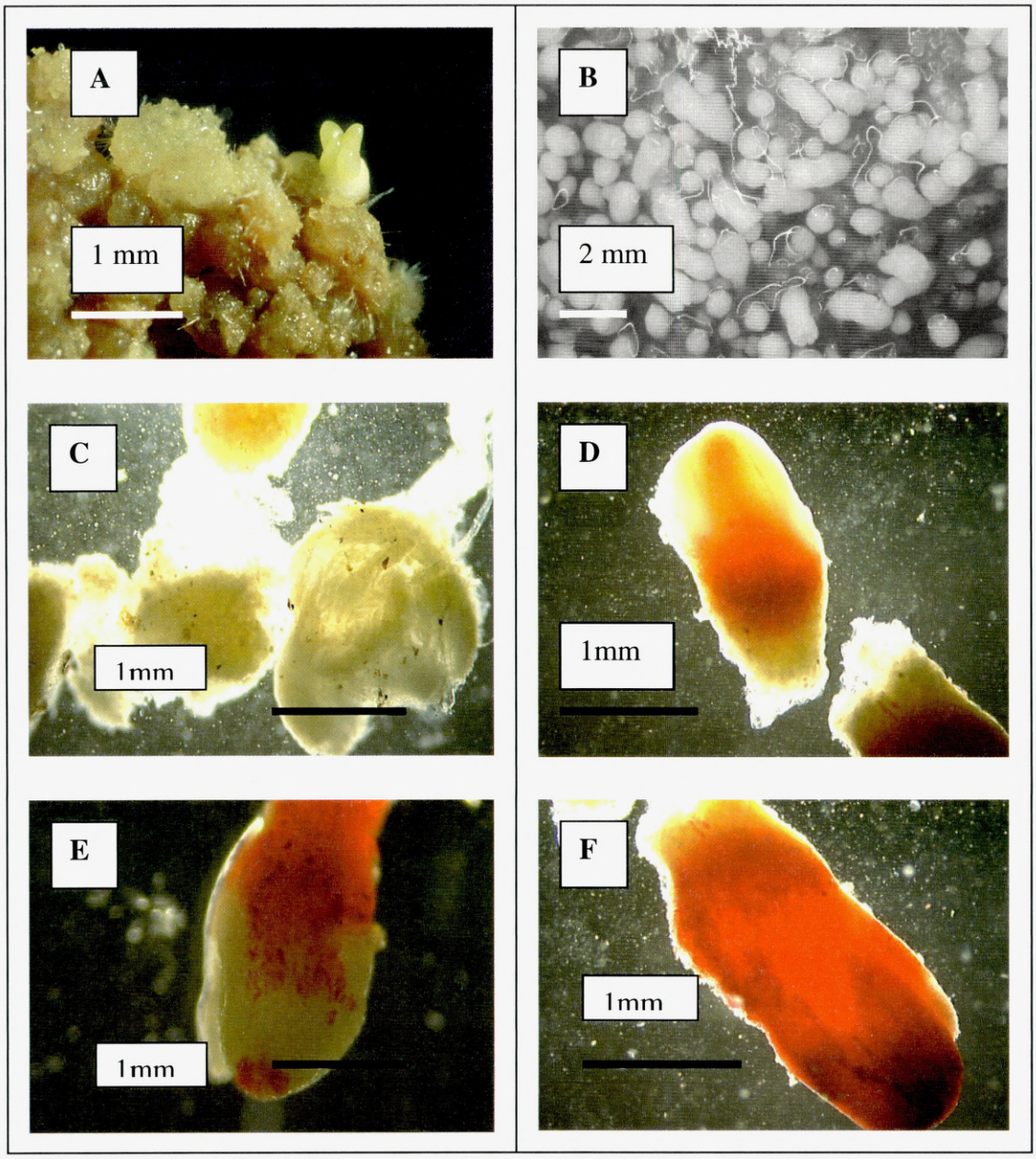


Figure 3.9 Images of somatic embryos

A-B Untreated somatic embryos of *P. sitchensis* at (A) singular mature early torpedo stage and (B) immature size 2 (I) globular, and mature size 3 (M) . **C-F Cryopreserved somatic embryos**, 48 hours after thawing and TTC incubation (Magnification x 50) (C) damaged immature globular somatic embryos (D) partial viability in mitotically active central region of a mature heart shaped embryo. (E) partial viability in mature torpedo to cotyledonary stage embryo. (F) full viability in mature torpedo to cotyledonary stage embryo.

Using optimised conditions for desiccation, cryopreservation investigations were applied and somatic embryo survival assessed after 14 days through TTC staining. Figure 3.11 a-c shows the number of embryos for each genotype (A1, A5 and C5), at each developmental stage (immature and mature) following treatment (control, 0.4M sorbitol + Na-alginate encapsulation, encapsulation + desiccation and all treatments + LN immersion) that showed 50% or more, red formazan cover following tetrazolium triphenyl chloride (TTC) infiltration. TTC survival was assessed following experiments undertaken in July (2003).

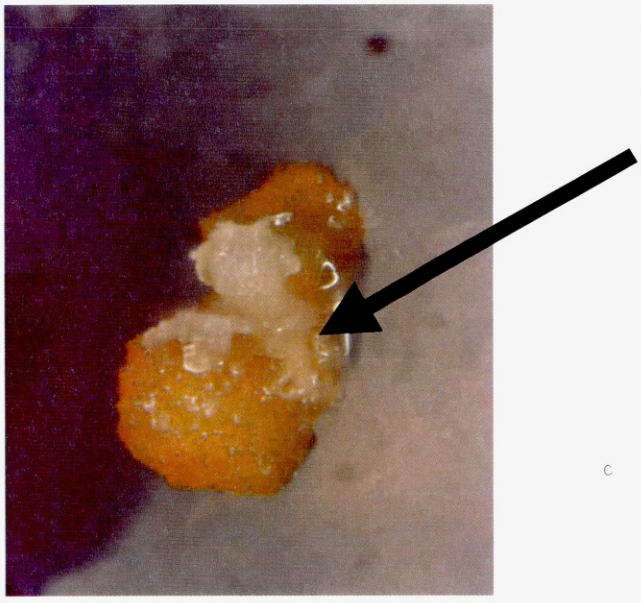


Figure 3.10

Image of NEM re-initiating from basal area of stage 2 globular, immature embryo.

General observations are: (1) following encapsulation survival improved for some genotypes after desiccation (2) for A1 and C5 survival was reduced between desiccation and LN treatments, indicating some LN injury , and (3) there was no reduction in survival for genotype A5 between control and post-LN; indicating that pre-cryo stages of the protocol largely influenced survival for this genotype. Following treatment the highest TTC response (20-50%) in all genotypes was observed in desiccated, size 2 embryos. Positive TTC survival (10-50%) was also observed following LN treatment in genotypes A1 and A5. C5 showed no response in control or post-LN treated embryos. There was variability (SEM) in the median survival in replicate plates (5-15%).

Cryopreservation investigations also examined recovery after 4 months; assessed as the number of somatic embryos showing embryogenic suspensor mass (ESM) and non embryogenic mass (NEM) re-initiation from the embryos for the same parameters as specified for TTC survival (Fig.3.12). Recovery was assessed for two experiments; the first undertaken in July (2003) and the second in December (2003). Table 3.1 summarises these responses without treatment and following LN immersion. NEM re-initiation is markedly greater than ESM re-initiation, but statistical analysis was required to determine the relative effects of

each factor (genotype, treatment). Statistical analysis (Table 3.2) was applied to compare the effects of genotype, embryo maturity, treatment and the month of the experiment on the proliferation of NEM (Table 3.2).

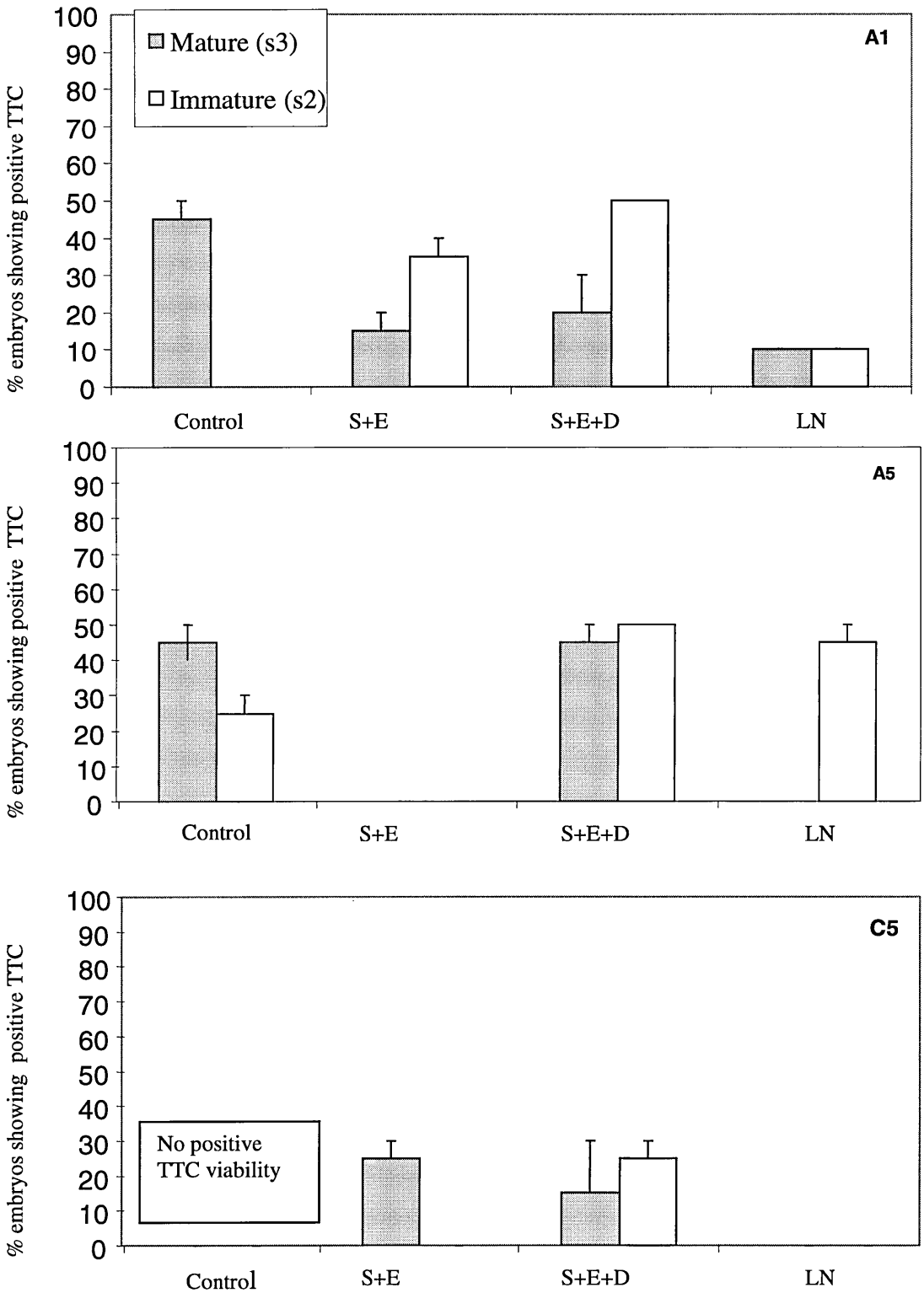


Figure 3.11. Percentage of embryos showing at least 50% TTC formazan product Encapsulation-dehydration treatments in genotypes: A1, A5 and C5. Data is median ± SEM of 5 replicates (beads) per treatment, per genotype, per experiment, experiment undertaken twice data recorded after 14 days. S= 0.4M sorbitol, E= Na-alginate encapsulation, D= 0.75M sucrose dehydration (18hr) and 4hr laminar air desiccation.

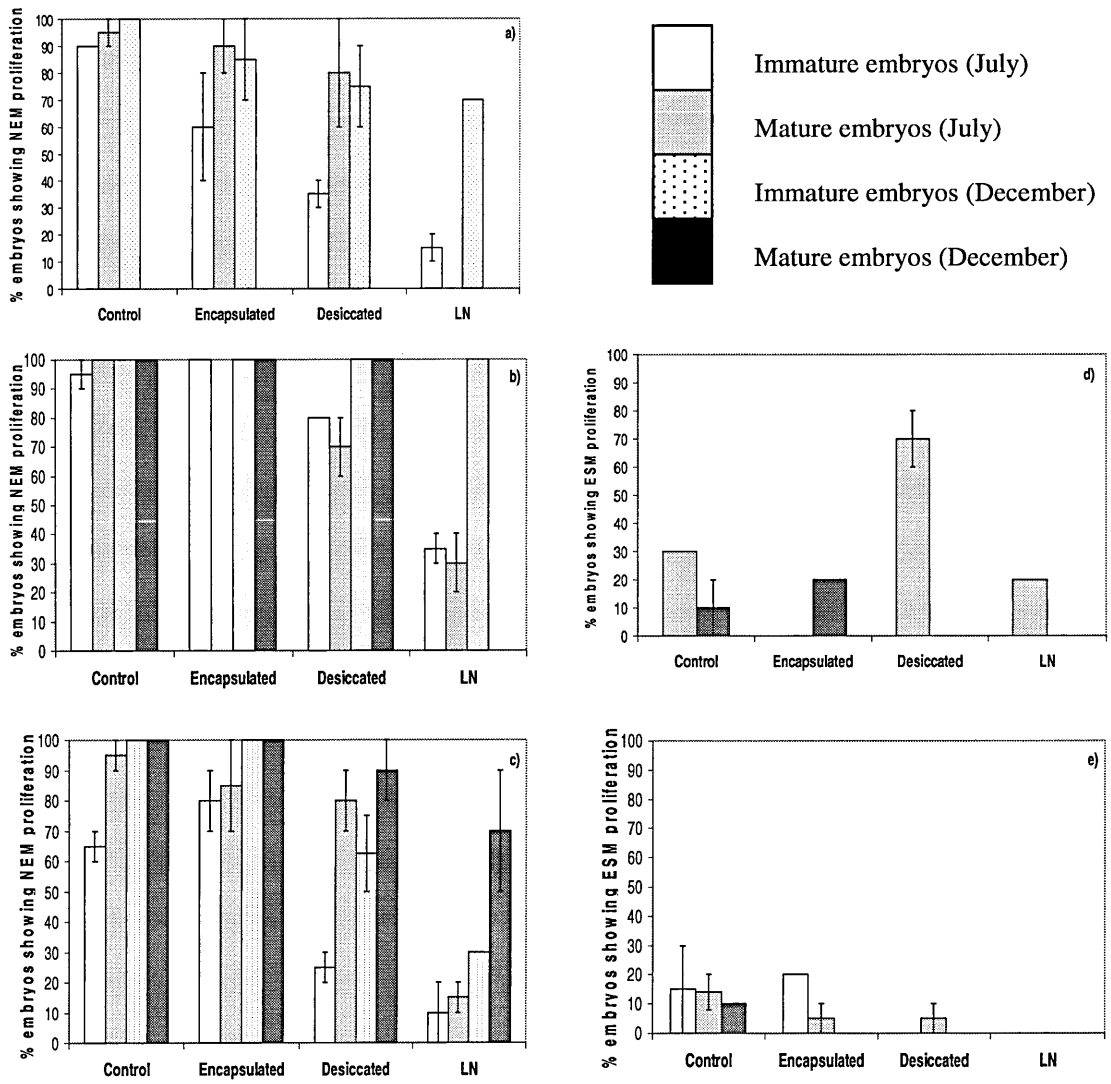


Figure 3.12 The effect of encapsulation-dehydration cryopreservation treatments on embryogenic suspensor masses (ESM) and non embryogenic masses (ESM)

Re-initiation from immature (size 2) and mature (size 3) somatic embryos sampled in July and December 2003.

The percentage of embryos 4 months after treatment:

NEM re-initiation from immature and mature embryos a) A1 b) A5 and c) C5

ESM re-initiation from immature and mature embryos d) A5 e) C5

Data are % means $\pm$ SEM of 10 replication, repeated twice. No ESM produced for genotype A1.

NEM data fulfilled normality assumptions and a General Linear Model was applied to compare the variance between factors and determine the level of significance each exerted on the proliferation of NEM. NEM data indicated that in some cases two factors (developmental state and genotype/month, treatment and month) acted together (interacted) at a significant level ($P<0.05$) to influence, proliferation. This can be seen in the Interaction plot (Fig. 3.14) where the line pattern varies compared to the others, such as the immature embryo point in genotype A5. ESM data did not fulfil normality assumptions and statistical analysis could not be progressed.

Table 3.1 Summary of the factors affecting ESM and NEM re-initiation recovery from somatic embryos

Genotype	**A1**				**A5**				**C5**			
Embryo Size	*2*		*3*		*2*		*3*		*2*		*3*	
Treatment	*Con*	*+LN*	*Con*	*+LN*	*Con*	*+LN*	*Con*	*+LN*	*Con*	*+LN*	*Con*	*+LN*
July	✓✗	✓✗	✓✗	✗✗	✓✗	✓✗	✓✓	✓✓	✓✓	✓✗	✓✓	✓✗
Dec	✓✗	✓✗	✗✗	✗✗	✓✗	✓✗	✓✓	✗✗	✓✗	✓✗	✓✓	✓✗

1st tick or cross represents proliferation non embryogenic mass (NEM)

2nd tick or cross represents Embryogenic suspensor mass (ESM) proliferation

Con= Control untreated embryo

+LN= Embryo pre-treated with 0.4M sorbitol for two days, 0.75M sucrose for 18hr, desiccated for 4hr and immersed in LN for 24hr prior to re-warming.

Size 2 embryos –'Immature' Size 3 embryos- 'Mature'

The factors affecting ESM and NEM re-initiation are shown in Table 3.1. Trends in ESM and NEM survival and re-initiation are highlighted using both graphical and statistical data. Somatic embryos from all genotypes, sizes 2 and 3, proliferated NEM and A5 (s3) and C5 (s2 and s3) proliferated ESM. The order of responsiveness in NEM re-initiation was A5>C5=A1 and in ESM re-initiation A5>C5>A1 (Fig. 3.12). All factors (month, treatment, genotype and developmental state) significantly ($P<0.01$) influenced NEM proliferation. ESM survival was influenced by the month, treatment and developmental state of the donor somatic embryo but the genotype of the somatic embryos mostly influenced the mean number showing ESM re-initiation.

Table 3.2 Statistical significance of influencing factors on NEM re-initiation from somatic embryos.

Factors	P value
Developmental State x genotype	P=0.02
Developmental State x treatment	P=0.026
Treatment x month	P=0.02
Month	P=0.000
Treatment	P=0.000
Genotype	P=0.000
Developmental State	P=0.01

Data fulfilled normality assumptions (most embryos showed some recovery) and a GLM analysis was performed on all data. There were no significant differences between replicate experiments.

Table 3.2 shows A5 embryos produced significantly more ($P<0.001$) NEM throughout treatments than genotypes C5 or A1. As embryos were subjected to increasingly more stressful treatments a decreasing trend in NEM proliferation was seen (Fig. 3.12) from a control range of 65-95% to a post-LN range of 10-35%. The effect of treatment was very significant ($P<0.001$). Mature embryos A1 and A5 showed a marked reduction (and loss in A1) in NEM production following LN (Fig. 3.12, Table 3.1). In genotypes A1 and C5 mature embryos produced significantly more ($P<0.01$) NEM than immature embryos, except for A5 where mature and immature embryos showed similar responses (Fig. 3.13). The month of the experiment was very significant ($P<0.001$); more NEM was produced in all embryos in December experiment compared to that in July experiment. The variability between replicate batches ranged from 0 to 20%.

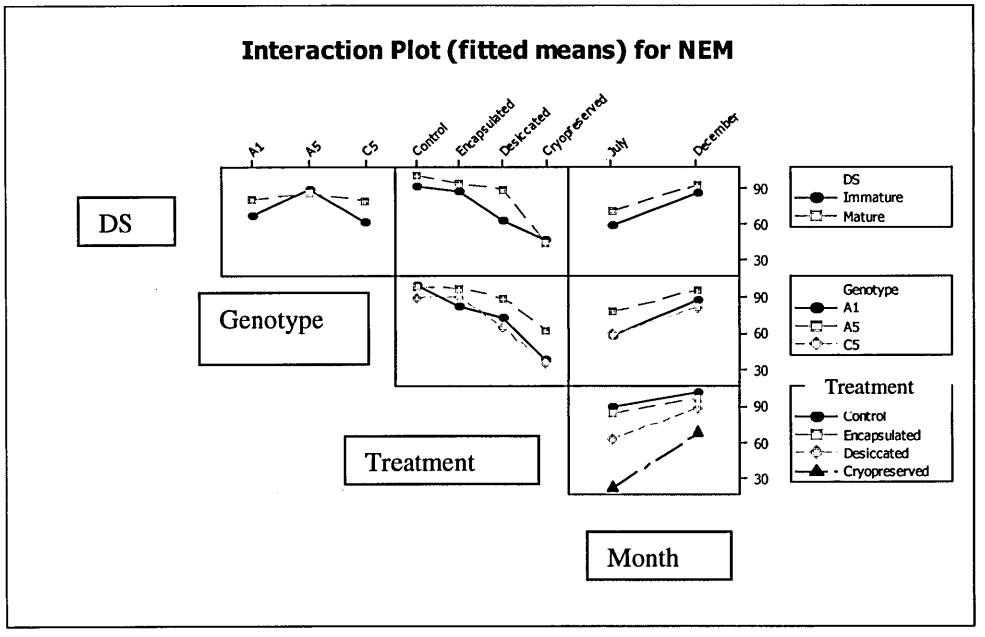


Figure 3.13 Effects of cryopreservation treatments on non embryogenic mass (NEM) proliferation
DS=Developmental State

There was a difference in ESM re-initiation between genotypes (Table 3.2), but not in survival. A5 showed the highest number of embryos re-initiating ESM (ca. 70% s3, desiccated) followed by A5 (20% s2, encapsulated). Genotype A1 showed no embryogenic capacity. Embryos produced the most ESM following desiccation in genotype A5 and following encapsulation in C5. The number of embryos showing re-initiation following desiccation (70%) in A5 embryos (Fig. 3.12) was over twice the number of the control (30%). Only mature embryos reinitiated ESM following desiccation and LN immersion (Fig. 3.12) and ESM was mostly re-initiated from the July (2003) cryopreservation experiment.

A scatterplot (Fig. 3.14) was plotted to determine if there was a relationship between ESM and NEM production; the line indicated that there was a linear relationship; the greater the number of embryos producing NEM, the greater the number producing ESM. To test if this was a statistically significant relationship, a Spearmans Rank (rs) correlation was applied. This test confirmed that there was a significant ($P<0.05$) relationship.

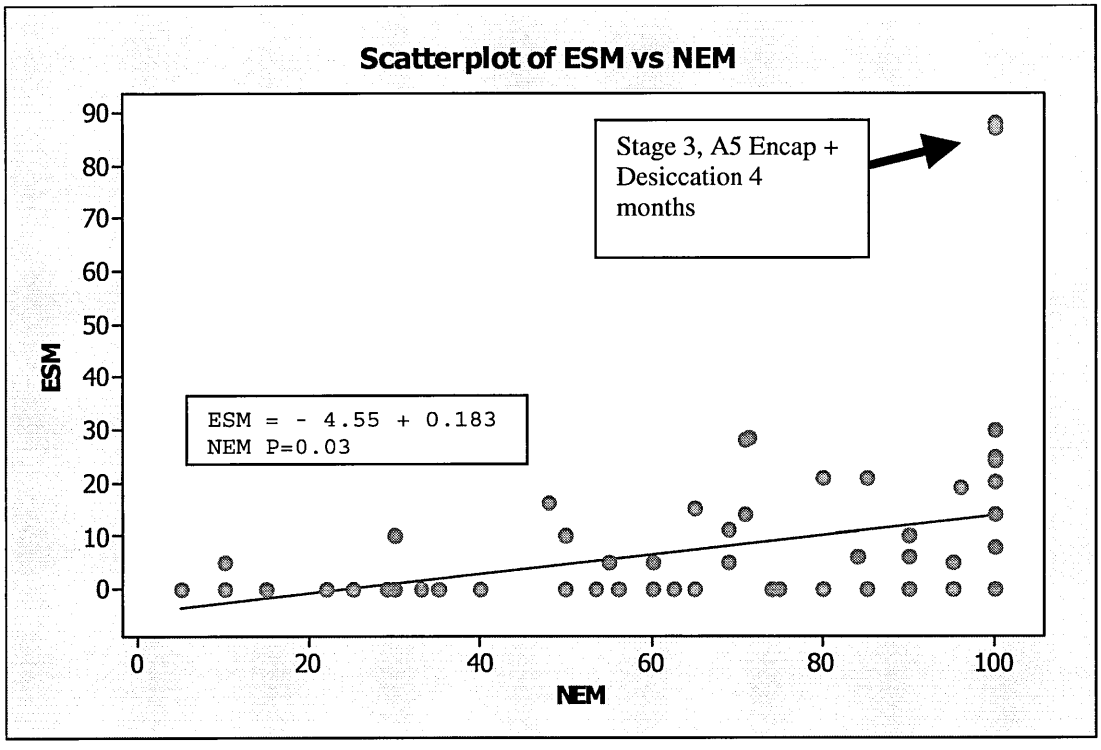


Figure 3.14 Scatterplot of embryogenic suspensor mass (ESM) vs non-embryogenic mass (NEM) production.

There was a significant ($P<0.05$) positive relationship between ESM and NEM. Each data point is the mean number of embryos producing callus from 20 replicates. An unusual data point is depicted by the arrow genotype A5, size 3 embryos that had been treated with sorbitol, Na-alginate encapsulation and 4hr desiccation.

3.3.2 Plant Vitrification Solution 2

A vitrification protocol was developed and successfully applied to somatic embryos of two genotypes (C5 and D5) at two developmental sizes [immature (2) and mature (3)]. The protocol comprised of a 2-day 0.4M sorbitol treatment, 2M glycerol and 0.4M sucrose loading treatment at 0°C for 60 min, Plant Vitrification Solution 2 (PVS2) incubation at 0°C for 30min and LN immersion.

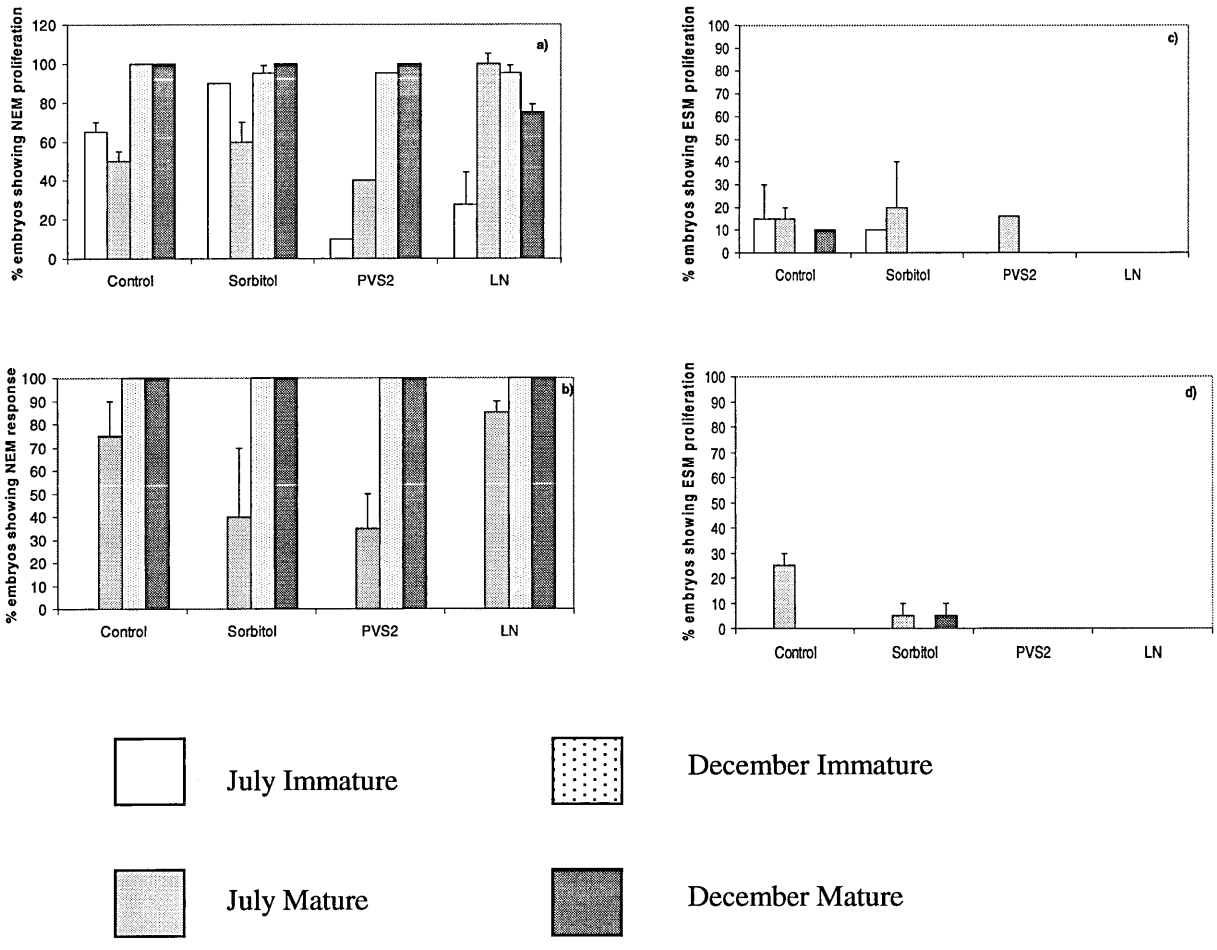


Figure 3.15 The effect of vitrification cryopreservation treatments on immature (size 2) and mature (size 3) somatic embryos sampled in July and December 2003.

NEM re-initiation for a) genotype C5; b) D5 (July immature somatic embryos not available) and ESM re-initiation for c) C5 and d) D5.

Experiments were undertaken in July 2003 and December 2003 and the results were assessed as the number of replicate embryos showing re-initiation (proliferation of ESM and NEM) four months after treatment (Fig. 3.15). Both genotypes re-initiated NEM although ESM did not following LN treatment.

A summary of the genotype/developmental size, control and post-LN ESM and NEM response to each treatment, at each experiment month is shown in Table 3.3. The statistical significance of each of the four main factors was tested using a GLM and is shown in Table 3.4. ESM data was not normally distributed (but was not skewed) and was transformed using an arcsine calculation, prior to GLM analysis; NEM data was normally distributed and a

GLM was directly applied. The main effects plots for ESM and NEM are shown as Figs 3.16 and 3.17.

Table 3.3 Summary of vitrification effects

Genotype	**C5**				**D5**			
Embryo size	2		3		2		3	
Treatment	Con	+LN	Con	+LN	Con	+LN	Con	+LN
July	✓✓	✓×	✓✓	✓×	××	××	✓✓	✓×
December	✓×	✓×	✓✓	✓×	✓×	✓×	✓×	✓×

1st tick or cross represents proliferation non embryogenic mass (NEM)

2nd tick or cross represents embryogenic suspensor mass (ESM) proliferation

Con= Control untreated embryo

+LN= Embryo pre-treated with 0.4M sorbitol for two days, Plant Vitrification Solution 2 –30min, and immersed in LN for 24hr prior to re-warming.

Size 2 embryos ='Immature' Size 3 embryos- 'Mature'

There was no significant ($P>0.05$) difference in genotype ESM and NEM proliferation between C5 and D5 (Table 3.4). Factors month and treatment significantly affected ESM and NEM proliferation ($P<0.05$), although genotype and developmental state did not ($P>0.05$). Variability between replicate batches was greatest (5-25%) in mature embryos tested in July 2003.

Table 3.4 Statistical significance of main factors on ESM and NEM proliferation

	Embryogenic suspensor masses (ESM)	**Non embryogenic masses (NEM)**
Statistical Test Applied	**Arcsine transformation and GLM**	**GLM**
Month	P=0.000	P=0.000
Treatment	P=0.000	P=0.062
Genotype	P=0.37	P=0.789
Developmental state	P=0.111	P=0.85

NEM untransformed (data fits normality assumptions), ESM variance was compared using a General Linear Model (GLM) in Minitab version 14.

The NEM response was similar between C5 and D5. NEM proliferated in the greatest number of embryos (80-100%) following all treatments applied to D5 and C5 embryos tested in December (Fig 3.15). The order of treatment recovery for both genotypes was control>sorbitol>LN >PVS2 (Fig 3.17) and this effect was statistically significant ($P<0.05$). In the July experiments recovery was reduced by 50% in immature and mature embryos treated with PVS2 (+ loading solution) (Fig. 3.15).

ESM re-initiation was observed for both genotypes following control and sorbitol treatments (5-25%) but only C5 reinitiated ESM following PVS2 treatment (Fig. 3.15), there was no post-LN recovery. Re-initiation was mostly observed in July-tested embryos and this was very statistically significant ($P<0.001$).

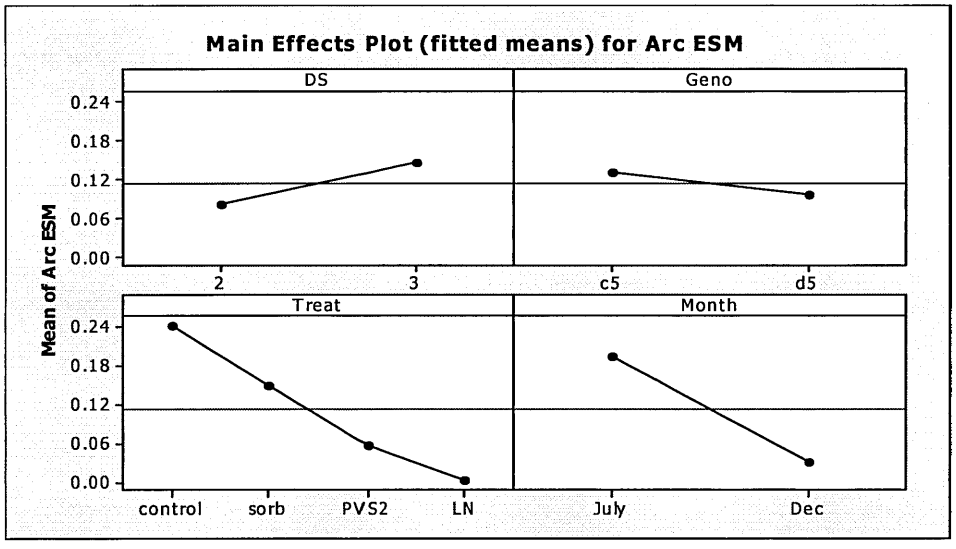


Figure 3.16 Main effects (no significant interaction) on ESM re-initiation from treated somatic embryos.

No significant difference between replicate experiments. N=10 replicates per experiment, undertaken twice. Treatment $P<0.05$; Month $P<0.05$. Central line at 0.12 represent Arcsine transformed % mean of somatic embryos showing ESM proliferation.

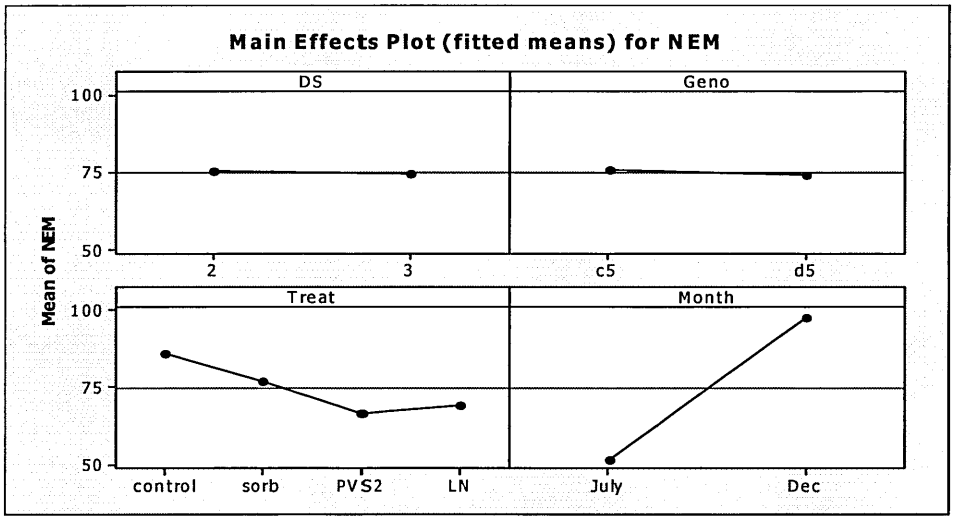


Figure 3.17 Main effects (no significant interaction) on NEM re-initiation from treated somatic embryos.

Data normal but transformed for comparison using Arcsine and General Linear Model performed in Mintab vs 13. No significant difference between replicate experiments. N=10 replicates per experiment, undertaken twice. Month $P<0.05$. Central line at 75 represents % mean of somatic embryos showing NEM re-initiation.

3.3.3 Encapsulation–vitrification (PVS2)

Although some replicates showed initial signs of recovery all replicates were subsequently contaminated during post-cryopreservation treatment and this study could not progress further in the time frame of the project and with the limited availability of somatic embryos.

3.3.4 Results summary

- Somatic embryos from five genotypes of two developmental stages were successfully reinitiated into ESM and NEM following two vitrification treatments: encapsulation-dehydration and Plant Vitrification Solution 2.
- An encapsulation-dehydration method was developed and a desiccation time of 4 hr (relative humidity 45% and temperature 20°C) was selected after optimising the % water content of beads (Fig 3.7 and post desiccation survival plot Fig 3.8).
    - TTC was used effectively as an early indicator of post treatment embryo survival and as a developmental indicator as to the location of enhanced mitotic activity (Fig. 3.9).
    - ESM was re-initiated from genotype A5 and C5 (Fig. 3.12) but not A1. The highest ESM re-initiation was from mature, desiccated A5 embryos (Fig. 3.12d). Statistical analysis could not be applied to ESM re-initiation data.
    - All factors (month, treatment, developmental state, genotype) were significant ($P<0.05$) in NEM re-initiation (Table 3.2). The greatest NEM re-initiation occurred in untreated, mature, A5 somatic embryos, tested in December 2003 (Fig. 3.13).
    - A statistical correlation test showed that there was a significant relationship between NEM and ESM re-initiation ($P<0.05$) throughout encapsulation-dehydration treatments (Fig. 3.14).
    - One genotype (A5) reinitiated ESM (20%) (Fig 3.12d), and all three genotypes (A1, A5 and C5) re-initiated NEM (10-70% depending on other factors), following LN immersion.

- A PVS2 protocol was developed for somatic embryos that included a 2hr 2M glycerol plus 0.4M sucrose 'loading 'treatment at 0°C, and PVS2 treatment for 1hr at 0°C.
    - ESM and NEM was produced from the two genotypes tested (C5 and D5).
    - The greatest ESM re-initiation occurred in untreated somatic embryos tested in July (Fig.3.15d and 3.16). The greatest NEM re-initiation occurred in untreated somatic embryos tested in December (Fig. 3.15b and 3.17).

- Statistical analysis was applied to both ESM and NEM re-initiation data, showing that the response of both was influenced by the experimental month and treatment, but not by the genotype or developmental state (Table 3.4).
- Notably, somatic embryos treated in the second experiment (December 2003) six months after the first experiment (July 2003), showed a significant ($P<0.01$) (Table 3.2) reduction in ESM re-initiation (Fig 3.11d-e).
- ESM did not re-initiate, but NEM did proliferate (20-100%), following LN immersion (Fig. 3.15).

In both methods, somatic embryos at stage 3 (more mature), mostly in the July 2003 experiment showed the greatest ESM re-initiation. The cryopreservation method of encapsulation–dehydration was more effective in ESM proliferation than PVS2 methodology applied in this study on the respective genotypes tested.

3.4 Discussion

Somatic embryos were cryopreserved through encapsulation-dehydration and vitrification (PVS2) protocols, and were subsequently reinitiated into dedifferentiated embryogenic suspensor masses (ESM) and non embryogenic suspensor masses (NEM) cultures that could be used to rapidly mass propagate elite Sitka spruce trees. The somatic embryo genotype, developmental size, *in vitro* age and treatment affected embryo survival and the number re-initiating ESM and NEM.

Current *in vitro* knowledge indicates that ESM provides a direct pathway in producing thousands of somatic embryos of specific genotypes, that develop into emblings and plantlets for use in clonal forestry. NEM cannot directly be used for embryo development but investigations have been undertaken in other species to determine the biochemical link between NEM and ESM proliferation. Twenty to thirty percent of mature somatic embryos of genotype C5, cryopreserved in July showed ESM proliferation. ESM proliferation was reduced in somatic embryos tested six months later, in December.

The discussion will now consider in more detail the development and application of each protocol, physiological and development aspects of somatic embryo re-initiation and treatment response, and the application of somatic embryo cryopreservation to Sitka spruce germplasm conservation in forestry and tree improvement programmes.

3.4.1 Encapsulation-dehydration

3.4.1.1 Preliminary investigations

Preliminary investigations were undertaken to (a) determine the response of somatic embryos to sorbitol dehydration and encapsulation over a 4-month test period, and to define reliable assessors of ESM recovery (Fig 3.6), and (b) optimise the laminar air-flow desiccation time and therefore bead moisture content prior to cryopreservation (Figs 3.7 and 3.8). Investigations were initiated based on the protocol developed for ESM (Chapter 2) and on encapsulation-dehydration protocols developed for other species (Dereuddre, *et al.,* 1991).

Summary

Over four months the embryos changed colour from white to beige to brown. At four months, dark browning was associated with the degradation of embryos and the loss of ESM development. Healthy ESM was separated from the donor embryo and placed in routine sub-culture. ESM re-initiation was observed in 20-30% of donor embryos and was greatest in control mature embryos. Sorbitol pre-treatment and encapsulation reduced ESM re-initiation two to threefold fold in mature embryos but increased twofold in immature embryos. The greatest ESM re-initiation was observed 3 months after cryopreservation.

Moisture content and survival, as determined by TTC analysis, indicated that 4hr of laminar-air desiccation of sorbitol dehydrated, encapsulated, sucrose incubated somatic embryos corresponded with 20-25% water content and 50-90% survival (TTC). No differences in water content were observed between immature and mature embryos and this protocol was selected for cryopreservation trials. Treatment factors and responses in the preliminary trials will be discussed.

(a) Experimental Factors

Sorbitol pre-treatment

In many plant species tested an osmoticum-based somatic embryo pre-culture is essential for a successful encapsulation-dehydration cryopreservation (Shibli, 2000, Shibli and Al-Juboory, 2000, Turner, *et al.*, 2000). Sorbitol was selected in this study because of the success in immature ESM cryopreservation testing (Chapter 2). The osmotic pre-treatment appears to be important in determining the embryogenic capacity of the resultant dedifferentiated cell mass. In non-encapsulated cocoa somatic embryos, a 3-week 6% sucrose basal medium incubation showed the highest percentage (80.5%) of secondary embryogenesis (re-initiation) (Agarwal, *et al.,* 2004). The sucrose pre-treatment may act not only as an osmoticum, but also as a

carbon source for somatic embryogenesis. Sorbitol has been reported as a pre-treatment osmoticum for the somatic embryos of *Pinus patula* (Ford, *et al.,* 2000), embryogenic cultures of *P. mariana* (Touchell, *et al.,* 2002) and embryogenic cultures of *Pinus roxburghii* (Mathur, *et al.,* 2003).

Na-alginate encapsulation

Encapsulation was crucial for somatic embryos to survive desiccation treatments; without which the protective bead embryos became shrivelled and were irreversibly damaged. These results are comparable to other encapsulation-dehydration trials. In carrot somatic embryos Na-alginate encapsulation was critical for post-dehydration survival and germination (Timbert, *et al.,* 1996). A detailed review of the benefits and functions of the encapsulate bead were discussed by Gray and Purohit, (1991). One factor discussed was the function of the bead as a seed coat assisting in gas exchange regulation. Interestingly studies of wheat (*Triticum aestivum* L.) cell cultures showed that a low oxygen concentration promoted embryogenic callus formation and suppressed non-embryogenic callus.

Laminar air-flow desiccation optimisation – moisture content and survival

A 4hr desiccation time was optimised from moisture content (25%) (Fig. 3.7) and TTC survival (Stage 2-55% and Stage 3 –90%) plots (Fig. 3.8). These results correlate well to other somatic embryo encapsulation-dehydration reports. In encapsulated black iris somatic embryos post-cryopreservation survival was observed in beads desiccated for 4-6hr (Shibli, 2000). Olive encapsulated somatic embryos also required 4hr of desiccation to acheive a moisture content of 21.1% ensuring cryopreservation survival (Shibli and Al-Juboory, 2000). Non-encapsulated *P. abies* somatic embryos can tolerate desiccation to 10% via silica gel or salt solution equilibration desiccation over 6 days (Gupta and Grob, 1995).

(b) Reliable assessors of Embryogenic suspensor mass recovery

Tetrazolium Triphenyl Chloride

TTC was used to assist in laminar air-flow desiccation optimisation. A positive TTC response in this study was determined 50% or more of the somatic embryo surface showing the red formazan product. Percy, *et al.*, (2001) determined embryos to be alive only if the entire embryo stained a deep pink, and dismissed those with irregular staining. However, secondary somatic embryogenic mass may occur from only a limited number of cells, as is observed in Fig. 3.10. The TTC stain has been reported to over-estimate survival after freezing (Ishikawa, *et al.,* 1995, Percy, *et al.,* 2001). Ishikawa, *et al.,* (1995) postulated that the discrepancy between TTC and growth in bromegrass suspension cells was due to excess electron donors in samples containing a mixture of both intact and injured cells, hence overestimation could

occur at only borderline viability. In this study TTC was useful as an early viability assessment criterion, but it is crucial to apply the test in combination with re-growth assessments.

Colour

There was no significant correlation between colour and ESM proliferation ($P>0.05$). Other studies have noted correlations between the colour of the donor embryo and the resultant dedifferentiated mass. There was no difference in somatic embryo colour response after different sorbitol and encapsulation treatments. In this study, colour could not be used as a reliable assessor of embryogenic capacity and recovery. The possible underlying physiological reasons for somatic embryo colour change will be discussed in depth in section 3.4.3.

Time and Developmental Stage

The encapsulation-dehydration protocol was applied to somatic embryos of both developmental stages (2 and 3). Untreated s3 embryos showed marginally more ESM re-initiation than encapsulated s2 embryos, but the optimised protocol can be applied to both developmental stages. ESM recovery was greatest in sorbitol and encapsulated treated embryos between 2 and 4 months. ESM harvesting should be initiated in this time frame.

3.4.1.2 Cryopreservation

An encapsulation-dehydration protocol was optimised for the cryopreservation of somatic embryos that incorporated a 2-day 0.4M sorbitol pre-treatment, encapsulation in 3% (w/v) Na-alginate beads, 0.75M osmotic dehydration for 18hr and laminar air-flow desiccation for 4hr. Embryo survival and recovery was determined through TTC imaging of intact embryos, after 14 days, and the survival and re-initiation of ESM and NEM cultures after 4 months. TTC indicated that the greatest viability (45%) was in A5 immature embryos (Fig 3.8). The greatest post-cryopreservation re-initiation (100 % NEM and 20% ESM) was observed in A5 immature embryos (Dec.) and mature embryos (July) respectively.

There are limited reports of encapsulation-dehydration applications to somatic embryos. Successful encapsulation-dehydration protocols for somatic embryos of olive and black iris species required a 4-day incubation in 0.75 to 1.25M sucrose– based medium prior to cryopreservation (Shibli, 2000, Shibli and Al-Juboory, 2000). Secondary embryogenesis was achieved in only 10% of embryos. Cryopreserved encapsulated coconut plumules regenerated the greatest frequency (80%) of post-thaw callus following 72-96hr 0.75M sucrose incubation

and 7-8hr silica gel desiccation where 30% moisture content was achieved. Embryogenic tissue (66.7%) was re-induced in cryopreserved non-encapsulated somatic embryos of *Picea* sp. following drying at 97% and rehydration at 100% RH for 12hr prior to re-induction (Bomal and Tremblay, 2000).

(a) Genotype

By applying the encapsulation-dehydration cryopreservation to several genotypes two half-siblings (A1 and A5) and one genotype from a different family (C5) it is shown that viability and some physiological development are possible. Additionally, where embryogenic tissue was re-initiated from treated somatic embryos it did appear comparable to parent embryogenic tissue of that genotype.

To assess the influence of genotype upon somatic embryo survival and successive physiological development each set of data results will be discussed. TTC viability was assessed as the percentage of embryos showing 50% or more, red formazan product after 14 days. A1 and A5 showed the best TTC viability and both showed up to 40% viability in immature embryos following desiccation. The TTC results showed a different trend compared to that of the ESM and NEM physiological development. C5 re-initiation also did not correlate well to TTC survival. Inconsistency between TTC viability and regeneration results has been observed in *P. glauca* somatic embryos following LN immersion, but this was accounted for as overestimation in viability due to excess electron donors in mixtures of intact and injured cells (Percy, *et al.*, 2000). The *P. glauca* observations may concur with LN immersion results from this *P. sitchensis* study and go some way to explain the overestimation in post-LN viability but it is still unknown why in this study, no TTC viability was observed in control somatic embryos that were capable of both NEM and ESM proliferation.

Although genotype A1 is derived from the same family as A5, no ESM re-initiation was observed and there was reduced NEM production. There was a very significant genotype difference ($P<0.001$) in NEM production. Other multi-clone and multi-family cryopreservation investigations undertaken on *P. glauca* somatic embryos (Park, *et al.*, 1994) showed the greatest post-cryopreservation recovery response variability existed between clones within families. In this case, variability was attributed to differences in culture quality rather than genetics. In this study a greater number of clones and families would need to be investigated to determine whether there was a genetic component in post-LN ESM and NEM response.

(b) Developmental size

Investigations of different embryo size upon survival and physiological development were undertaken. Size 2 globular embryos (0.5 x 0.5 mm) were compared with size 3 cotyledonary (heart-shaped to torpedo) embryos (0.5-1.0 x 0.5-1.0mm). Size 3 embryos showed post-cryopreservation ESM re-initiation, but immature size 2 embryos showed superior TTC viability, possibly because they are anatomically more meristematic and less vacuolated.

Shibli, *et al.*, (2000) determined that olive embryos with a sized 2-4mm showed greater survival than smaller (1-2mm) or larger embryos. Tesserau, *et al.*, (1994) determined a relationship between the size of carrot somatic embryos and their viability after LN, with larger embryos showing less conversion to plantlets than smaller embryos (0.25-0.5mm). Larger embryos may be more vacuolated and therefore contain more water that could be detrimental during LN immersion. Results from relative moisture trials (Fig. 3.8) of encapsulated size 2 and 3 somatic embryos undertaken during laminar air-flow desiccation do not suggest that the larger encapsulated embryos contain more water.

In Sitka spruce somatic embryos, developmental size was clearly important in initial TTC response, and was significant for both somatic embryo survival after four months and NEM re-initiation ($P<0.05$). There are physical size and water-related parameters, which may be thermodynamically favourable but physiological factors that may be, necessary for embryogenic tissue re-initiation (section 3.4.3). In this study ESM re-initiation was shown not to be significantly influenced ($P>0.05$) by the developmental size.

(c) Month

The physiological development of ESM and NEM from treated somatic embryos was observed from experiments undertaken 6 months apart. The aim was to investigate the influence of the *in vitro* age of the original embryogenic tissue that was used to produce the test embryos. This factor and its effects on cryotolerance have been discussed previously with regards to immature dedifferentiated somatic embryogenic tissue (Chapter 2). Fig. 3.12 shows that only embryos cryopreserved in July were capable of regaining embryogenic capacity following desiccation and LN. More ESM is re-initiated from embryos tested in July and more NEM is produced in embryos tested in December. Somatic embryos cryopreserved in July were matured from embryogenic tissue that had been in culture for 3 yrs (36 subcultures). Embryos cryopreserved in December were matured from embryogenic tissue cultured for 3 ½ yrs (42 subcultures). The percentage of ESM re-initiation in December–tested control embryos (A5 and C5) was half that of equivalent July–tested embryos. The freezing tolerance of carrot somatic embryos varied depending on the age of the source

embryogenic cell strain although there was no negative correlation between the age of the strain and embryo survival over 32 successive subcultures (Tessereau, *et al.*, 1994).

The protocol may be improved in several ways. The incorporation of an initial robust viability test would indicate the post-cryopreservation survival status. The factors, which determine the post-cryopreservation survival of the entire embryo, may then be separated from the factors that determine ESM re-initiation. DSC will be applied to confirm protocol optimisation and determine where problems may arise (Chapter 5).
The direct maturation and germination of cryopreserved embryos is also required if synthetic seed is to be successfully generated. The optimal factor combination for ESM re-initiation in this study was the combination of cotyledonary size 3 embryos, derived from <3yr old *in vitro* embryogenic donor tissue.

3.4.2 Plant Vitrification Solution 2

A PVS2 vitrification protocol was optimised for the cryopreservation of somatic embryos that incorporated a 2-day 0.4M sorbitol pre-treatment, 2hr 0.4M sucrose and 2M glycerol treatment at 0°C and 30min PVS2 solution at 0°C. Embryo survival and recovery was determined through the re-initiation of ESM and NEM cultures. Size 2 and 3 somatic embryos were tested in July and December 2003. ESM re-initiation was observed in mature somatic embryos tested in July, in genotype C5 following PVS2 and in genotype D5 following sorbitol treatment. NEM re-initiation was observed in all treatments. In all cases ESM and NEM re-initiation was significantly ($P<0.05$) influenced by experimental month and treatment and not by genotype and developmental state. Somatic embryos of genotype C5 showed 20% recovery following PVS2 incubation but none following LN immersion.

Post-LN recovery has been achieved in non-encapsulated somatic embryos using PVS2 methodology for the monocotyledon *Macropidia fuliginosa* (Turner, *et al.*, 2000). A 2-day, 0.4M sorbitol or 0.8M glycerol pre-treatment/osmoprotection stage followed by PVS2 incubation prior to LN produced high survival (90.6%) of embryos. Further optimisation of PVS2 –based cryopreservation pre-treatment and osmoprotection for the *P. sitchensis* somatic embryos may improve post-LN recovery.

Treatment was very significant ($P<0.001$) for both ESM and NEM re-initiation. The main effect plot (Fig. 3.16) shows an almost direct negative correlation between the mean ESM re-initiation and the increasing treatment combinations (control– sorbitol – PVS2–LN). This response was paralleled for NEM production. This is a very different response to

encapsulation-dehydration where the re-initiation of ESM increased by a mean of 40% following desiccation. Somatic embryos did show a reduction in NEM proliferation following PVS2 treatment but only following the July experiment. Conversely, post-PVS2 treatment ESM proliferation was only observed in C5 embryos tested in July. Therefore in optimal explants PVS2 toxicity does not appear to be a limiting factor in PVS2 cryopreservation methodology.

In this experiment, there were no significant genotype or developmental effects ($P>0.05$) in ESM or NEM production. D5 and C5 genotypes showed very similar responses following control treatment as well as through pre-cryopreservation treatments. The fact that there was very little difference in ESM/NEM response between embryos of different developmental sizes during PVS2-based vitrification, but that there was, during encapsulation-dehydration-based protocols indicates that desiccation tolerance was altered during developmental maturation. The *in vitro* age of the donor culture was very significant ($P<0.001$) as it was in the case for encapsulation-dehydration.

3.4.3 Physiological comparisons

3.4.3.1 NEM and ESM – re-initiation physiology

The physiological development pathway of ESM and NEM re-initiation is not well understood, but the two types of dedifferentiated cell masses have been identified and characterised in other plant and conifer studies, although comparisons in relative production have not. Type A and B somatic cell masses were characterised in *P. abies* cultures (Egersdotter and Von Arnold, 1992). 'A' cell cultures are described as being isodiametric with a high nucleus/cell volume ratio, densely staining nuclei and nucleoli and leading to the development of somatic embryos. 'B' cells contained less dense cytoplasm and many small vacuoles. No mitotic activity was detected in these cells over the observation period (Kristensen, *et al.*, 1994). These non-embryogenic cells became progressively more vacuolated and elongated. Histological examinations revealed that there was no mitotic activity in non-embryogenic cells. Svodoboda, *et al.*, (1999) also noted embryogenic and non-embryogenic cell masses, in *P. abies* cultures. Non-embryogenic cell masses contained 'only a few loosely aggregated cells in embryonic regions.' Similar observations were noted in shoot-forming and non-shoot forming tobacco callus (Dhaliwal, *et al.*, 2003).

In this study, there was a significant correlation ($P<0.05$) between NEM and ESM re-initiation from *P. sitchensis* somatic embryos. Further research is required in this area to determine if NEM may be converted to ESM and if it can be converted without compromising

genetic stability. Investigations using transgenic cherry rootstock suggested that morphogenetic callus can be reverted to non-morphogenetic callus (Gutierrez and Rugini, 2004). Dedifferentiation can induce more genetic changes than regeneration through a differentiated route and may be associated with somaclonal variation problems (Harding, *et al.*, 1996, Scowcroft, 1984).

3.4.3.2 Role of dehydration/desiccation on embryos

In this study the proliferation of embryogenic tissue from somatic embryos was improved by dehydration and desiccation. Carbohydrates are used routinely in *in vitro* conifer culture maintenance for induction, proliferation and embryo maturation (Norgaard, 1997). Non-osmotic water stress is achieved by adding high molecular weight compounds such as polyethylene glycol, which do not penetrate the cell wall.

However, sugars do not simply act as osmoticum, but can also provide a carbon source for secondary embryogenesis (Agarwal, *et al.*, 2004). Carbohydrates have been shown to affect development *P. mariana* (Tremblay and Tremblay, 1995).

3.4.3.3 Physiological differences of developmental size

In this study size 2 and 3 somatic embryos showed differences, sometimes significant, in response to cryopreservation treatments. Biochemical profiling has shown that during somatic embryo development there is an increase in storage protein deposition, uptake of NH_4 and alterations in amino acid profile (Thorpe, 2000, 2004) as protein synthesis changes from a developmental to a germinative mode. ABA also promotes lipid accumulation in *P. glauca* (Attree, *et al.*, 1995) and ABA was used in the *P. sitchensis* maturation protocol. Dussert, *et al.*, (2001) showed that increased lipid content in the endosperm of coffee species seeds was associated with a decrease in osmotically inactive water. This water is thought to be important for cells to re-gain function following re-warming. This physiology correlates with the TTC results obtained in this study which show post-cryopreservation survival favouring size 2 embryos, but not with ESM re-initiation where size 3 embryos show a greater response. It has also been suggested by Tessereau, *et al.*, (1994) that post-cryopreservation survival was reduced in larger embryos because increased cellular differentiation makes controlled osmotic dehydration more difficult, with the optimal water status only achieved in some cell types.

3.4.3.4 Somatic embryo colour

It was hypothesised that the colour of the embryos may be a morphological indicator of survival and viability. A statistically significant correlation ($P<0.05$) was not observed in this

study, although a colour change from white to beige to brown was noted in post-treatment deteriorating embryos. Other researchers have noted colour to be associated with living or dead embryos. Svobodova, *et al.*, (1999), associated browning in *P. abies* somatic embryos with phenolic accumulation, damaged embryos and altered morphology. Phenolics were located in the surface layers of embryos where 'callusing' originated. Biochemistry tests showed subcellular compartmentation of condensed tannins in vacuolar deposits. In contrast it was noted in olive somatic embryos that only the brown embryos re-grew after cryopreservation, the cream or white embryos were dead (Shibli and Al-Juboory, 2000). The application of phenolic detection testing would clarify the origin of the discolouration observed during post-treatment embryo development.

3.4.3.5 *In vitro* age

In this study the age of the *in vitro* parent ESM culture, appears to have been a factor in the ability of subsequent embryos to re-initiate ESM and cultures that were 6 months younger showed greater proliferation following treatment components of both cryopreservation protocols. The effects of *in vitro* age related stress on ESM cultures, embryogenic capacity, the quality and number of subsequent matured somatic embryos and implications for cryopreservation have been discussed in Chapter 2 (see section 2.4.5). Following this investigation it was determined that *in vitro* aged parent ESM cultures also affect the regenerative capacity of subsequent embryos and may be one of the most significant factors in post-LN recovery. Aged parent cultures may lead to a range of epigenetic and chromosomal variations that affect subsequent development in clonal offspring. The frequency of aneuploid and hyperdiploid cells in onion and garlic suspension cultures increased with callus age (Mukhopadhyay, *et al.*, 2005).

In vitro aged plants may be under increased oxidative stress and free radical attack (see Chapter 1 General Introduction). There is evidence that oxidative stress and DNA methylation play a role in embryogenesis. Antioxidants were shown to have a negative effect on the initiation of embryogenic cultures, somatic embryo production and plantlet recovery (Malabadi and Van Staden, 2005). Rice embryogenic cells, with a higher antioxidant status and a lower pro-oxidant level (lipid peroxidation), showed greater post-cryopreservation survival than non-embryogenic cells (Lynch, et al., 1995). Comparisons in the *in vitro* age effects of the three explants ESM, somatic embryos and shoot-tips investigated in this thesis will be made in Chapter 7.

3.4.4 Applications to forestry

The cryopreservation of somatic embryos through encapsulation-dehydration offers an alternative route to Sitka spruce germplasm storage, and may circumvent the need for expensive programmable cooling equipment. LN storage of somatic embryogenic suspension cultures is now an integral component of conifer improvement programs
(Cyr, *et al.*, 1994, Cyr, *et al.*, 2001) The encapsulation of conifer somatic embryos as a means of cryopreservation is in preliminary stages of investigation and application. Somatic embryo cryopreservation would provide a cost-effective storage strategy that may be automated with a bioreactor, with no need for a programmable freezer. The choice of synthetic seed direct germination or multiplication through embryogenic tissue re-initiation, would meet market requirements, which may be the production of hundreds of thousands of clonal emblings within months of recovery from storage.

3.5 Conclusions

Encapsulation-dehydration can be successfully utilised to cryopreserve *P. sitchensis* mature somatic embryos and is reported here for the first time. Embryos were also able to survive the toxicity of the PVS2 application. *P. sitchensis* re-initiation is reported for first time and further work is required to understand NEM and ESM physiology and biochemistry. Genotype differences were observed and these appear to be most significant between clones of the same family. Another critical factor determined in this study was *in vitro* age; changes in morphogenetic competence were observed following 36 subcultures. The developmental maturity also determined somatic embryo post-treatment development. ESM was mostly re-initiated from mature embryos. Desiccation tolerance and water relations will be further investigated using thermal analysis Chapter 5.

Chapter 4 A PHYSIOLOGICAL AND BIOCHEMICAL STUDY OF *P. SITCHENSIS* SHOOT-TIP CULTURE CRYOPRESERVATION

4.1 Introduction

The aim of the research in this chapter is to progress the development of a cryopreservation protocol for *in vitro* shoot-tip apices of *P. sitchensis.* To date conifer shoot-tips have proven highly recalcitrant to both *in vitro* culture and cryopreservation. Therefore, the physiological basis for recalcitrance in this germplasm will be explored in this study. *In vivo* protection mechanisms (cold hardening and acclimation) utilised to simulate adaptation mechanisms and assist in the development of cryopreservation protocols will be emphasised. Markers of stress recalcitrance and DNA methylation established in previous studies of *in vitro* and cryogenic recalcitrance will also be used to enhance an understanding of stress in Sitka spruce shoot-tip cryopreservation. These markers will also assist in the future development of improved cryopreservation methods by pinpointing these critical factors and steps attributed to storage recalcitrance. Throughout the investigation emphasis will be placed on further development of methodologies that are technically amenable to genebanking practice in larger scale germplasm repositories.

4.1.1 *In vitro* shoot cultures in temperate woody species

Woody plant species are often difficult to culture and *in vitro* stress, ageing and recalcitrance play a significant role in limiting their manipulation *in vitro* (Benson, 2000). Plant tissue recalcitrance has been defined as, 'the inability of plant cells, tissues and organs to respond to tissue culture manipulations'(Benson, 2000). Investigations of *in vitro* stress are important in the context of this study because post-cryopreservation success may be compromised if physiologically stressed explants are incorporated in storage parameter testing. Conifer shoot-tip cultures exhibit particular problems during initiation *in vitro* largely because they have evolved *in vivo,* to occupy niches of extreme seasonal variation and are unable to maintain a continuous growth rhythm essential for *in vitro* utilisation. Their physiological status is impacted by the maturation and juvenility phases typical of long-lived woody perennials. Furthermore, their stress and adaptive physiologies are moderated by programmed and induced dormancy.

4.1.2 Conifer shoot-tip cultures in germplasm conservation and utilisation

The combination of the difficulties of conifer *in vitro* shoot-tip culture and, the relative ease of ESM and somatic embryo manipulation and cryopreservation has precluded a large amount of conifer *in vitro* shoot-tip research from being undertaken.

However, the Northern Research Station has successfully developed and established the effective propagation of vegetative *P. sitchensis* cultures (John and Murray, 1981) with proven field trial performance and with the application of a range of new cryopreservation techniques available (Benson, *et al.*, 2006 In press) recalcitrance may be overcome. Developments in the fundamental ecophysiology of conifers and cold acclimation methodology are now available (Havranek and Tranquillini, 1995) to assist in the development of novel cryopreservation methodology. The promise of encapsulation-dehydration/vitrification techniques may lead to facilitated automation and therefore increased economic viability making *in vitro* shoot-tip culture more appealing to industry and genebankers.

4.1.3 Conifer physiology

(a) In vivo

Boreal and temperate conifer species, in particular, show acute seasonal shoot growth variation, characterised by over wintering dormancy and strong episodic flushes during the growing season. Growth ends abruptly in the autumn, induced through shortened photoperiods and lower temperatures (between 10 and 20 °C) in preparation for subfreezing temperatures (<-30°C), (Havranek and Tranquillini, 1995). Winter hardiness is maintained until a prolonged warm period, usually in spring, occurs allowing growth to resume. Characteristics of full winter dormancy include resting buds, suspension of growth processes (elongation and divisional growth), a reduction in metabolic activity, frost and desiccation resistance enhancement and changes to cellular and cytoplasmic features.

The changes during cold acclimation occurring both at the cellular level and physiological manifestations of dormancy appear to be in part due to a genetically based endogenous rhythm (Mellerowicz, *et al.*, 1992). This can be illustrated experimentally in 'common' garden experiments and in climate chambers. Winter pine seedlings kept in climate chambers from autumn through winter, under constant long-day photoperiods and a constant temperature of 15°C still showed a decrease in photosynthetic capacity (Bamberg, *et al.*, 1967). Conifers are known to have a high level of genetic variation and it is possible that

genotype explants will exhibit a similar level of variation. The degree of differentiation is likely to be more acute in more developed explants such as shoot-tips.

(b) In vitro

The *in vivo* variability and strong endogenous rhythm, of conifer cultures often influences the initiation and maintenance success *in vitro* vegetative propagation. The initiation of *in vitro Pinus sylvestris* shoot-tips thus has only been possible in explants collected in January and April and not during autumn (Kuoksa and Hohtola, 1991).

Seasonal growth fluctuations (episodic flushes, monopodial shoot growth) are often continued through *in vitro* culture. Seasonal patterns of *in vitro P. sitchensis* cultures have been observed (Selby and Harvey, 1985) depending on the time of year explants are procured with periods of inactivation leading to culture deterioration.

Another problem particular to woody plants is that when explants, obtained for *in vitro* initiation, are taken from mature trees the resultant shoot-tip cultures often deteriorate rapidly or exhibit abnormalities such as plagiotrophic (grows like a branch, not apically), growth and desynchronised bud flushing as was reported for *Larix deciduas,* (Bonga, 1989).

4.1.4 Markers of *in vitro* storage recalcitrance

The development of diagnostic markers for stress is one of the possible methods to investigate plant *in vitro* recalcitrance and may elucidate the physiological and molecular factors that limit tissue culture and cryostorage responsiveness.

Whereas *in vitro* recalcitrance may be in part due to the genetic pre- determinism of the explant (Mccown, 2000), a number of *in vitro* plant recalcitrance problems are associated with one or more culture physiology related factors determined and measured by researchers. These factors include (a) oxidative stress that produces free radicals, lipid peroxides, and associated toxic aldehydic lipid peroxidation products and ethylene (Benson and Bremner, 2004) which may be determined by antioxidant assays (Johnstone, *et al.*, 2005 In press) and gas chromatography (Benson, *et al.*, 2006 In press); (b) plant maturation and degeneration processes associated with changes in DNA methylation patterns (Harding, *et al.,* 1996) and (c) overall plant vigour and health as measured by protein content (Bradford, 1976).

In plants, where light, water and oxygen and respiratory cycling are the driving forces of survival, free radical damage is largely generated by an imbalance in primary metabolic regulation leading to oxidative stress, and consequently this will be the first factor to be

considered. However, *in vitro* culture is supported by heterotrophic growth through the addition of organic carbon, specifically sucrose.

4.1.4.1 Oxidative stress: study rationale as applied to Sitka spruce

There are several different types of free radicals produced during metabolism. The hydroxyl radical (·OH) is considered the most reactive, and is generated from a series of reactions. Chemically, the most common source of hydroxyl radicals biologically is from Haber-Weiss and Fenton chemistry as shown in Fig. 4.1. Plant systems produce hydrogen peroxide from a number of pathways, and with the addition of ferrous ions, hydroxyl radicals may be readily generated. Enzymes may produce hydrogen peroxide directly and are the cause of superoxide dismutase (SOD).

Hydroxyl radicals can react with lipid membranes and produce further damaging, cytologically, active lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxy-2-noneneal (HNE) and end-products such as ethane. Hydroxyl radicals readily react with DMSO to produce the methyl radical and methane. Methane and ethane can be detected using gas chromatography (GC) and therefore be used as indirect indicators of oxidative stress (Benson, *et al.*, 2006 In press).

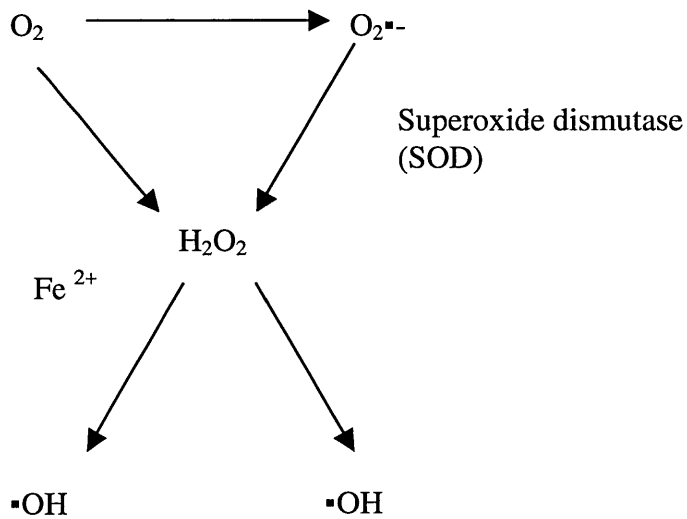
Figure 4.1 Oxidative stress reactions

a. Fenton reaction pathway of hydroxyl radical production (Benson and Bremner, 2004)

H_2O_2 + Fe^{2+} ⟶ ·OH + Fe^{3+} + HO^-

Hydrogen peroxide	Ferrous ion		Hydroxyl radical	Ferric ion		Hydroxide ion

b. Reactive oxygen species generation from O_2, (Benson and Bremner, 2004)



(a) Antioxidants

Antioxidants reduce oxidative stress in biological systems by scavenging free radicals and/or by removing reactive oxygen species. A number of researchers have proposed, that oxidative stress may be involved with *in vitro* responses (Adams, *et al.*, 1999, Benson and Roubelakis-Angelakis, 1992). Oxidative stress is associated with many factors of *in vitro* plant growth including: heterotrophic nutrition, artificial light, and reduced ventilation. *In vitro* manipulations, such as culture initiation and long-term sub-culturing, have been specifically associated with the production of active oxygen species (Benson, 1999b).

Antioxidant investigations have been previously applied to *in vitro* woody plant callus of *Vitis vinifera* (Benson and Roubelakais-Angelakis, 1992), where it was determined that a reduction in fluorescent oxidation products, in the latter stages of callus induction was concomitant with increased antioxidant activity. Antioxidant assays provide indirect methods to measure oxidative stress status. As cells have several antioxidant defence mechanisms, an assay was selected for this study that measured total aqueous antioxidant activity. This comprised an ABTS (2,2'-azinoibis-(3-ethyl-bezothiazoline)-6-sulfonic acid) scavenging assay developed

by Johnstone, *et al.*, (2005 In press). In this assay, antioxidant activity is determined spectroscopically as the antioxidant-induced scavenging of the pre-formed ABTS radical is reduced, from $ABTS^{\cdot+}$ to ABTS, and coupled to a decrease in absorbance at 734nm. The extent of the reaction is dependent on the level of antioxidant activity, concentration of the antioxidant and the duration of time the reaction occurred over. This assay was suitable for this study because large amounts of tissue were not required (limited shoot-tip cultures being available), and information was rapidly provided on the total antioxidant status of this system. This assay is an excellent primary assay for screening samples for an overall effect, from which more specific antioxidant studies can be undertaken.

Total antioxidant status studies were combined with evaluations of total protein, which may provide an indication of growth/vigour of shoot-tip cultures. The calorimetric protein assay based on the Coomassie method (Bradford, 1976), was selected for application as this is the assay of choice for plant tissues.

4.1.4.2 Ethylene

The stress-associated hormone ethylene is particularly likely to accumulate in the *in vitro* plant culture vessel environment and may have an acute effect on the growth and development of the system. Ethylene can inhibit *in vitro* plant growth and development and it promotes senescence. Some studies have shown that although ethylene may promote callus proliferation it may also inhibit shoot-tip growth (Biddington, 1992). In *Ficus* tissue cultures the more efficiently sealed culture vessels resulted in reduced leaf expansion, but the effect was reversed when explants were treated with an ethylene absorbent material 'Ethysorb,' (Jackson, *et al.*, 1991). In other studies ethylene has been reported to increase shoot formation; in *Pinus radiata* the presence of ethylene and CO_2 were necessary for shoot buds to differentiate on cotyledon explants (Kumar, *et al.*, 1987).

(a) Ethylene Production

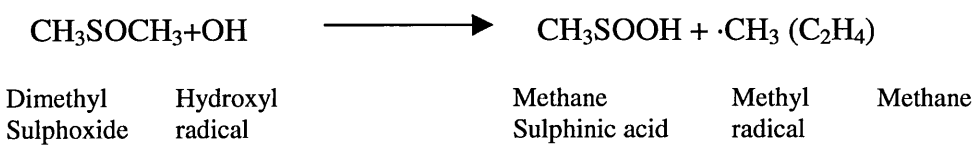
Ethylene is produced as an endogenous plant hormone and can be evolved exogenously from tissue culture medium, especially when other plant hormones such as auxins and cytokinins are included. Ethylene is also a decomposition product of lipid peroxidation, as •OH attack CH_2 groups of polyunsaturated fatty acids, generating lipid free radicals and eventually lipid hydroperoxide (Benson and Withers, 1987). The main source of hormonal ethylene is via 1-aminocyclopropanecarboxylic acid (ACC) synthase which converts S-adenosylmethionine (SAM), to ACC, the main precursor of ethylene (Biddington, 1992). ACC-dependent ethylene

biosynthesis is dependent on the lipid membrane integrity and a reduction in ethylene in *in vitro* stressed plant in this case may indicate that membrane damage has occurred.

(b) Ethylene and volatile marker detection

Ethylene can be analysed by volatile headspace sampling and gas chromatography (GC) (Benson, *et al.*, 2006 In press) along with other small-chained carbon products of lipid peroxidation such as methane, ethane and pentane. In combination with GC analysis, DMSO may be used as a hydroxyl radical probe (Benson and Withers, 1987) as methane is produced during the DMSO scavenging of the hydroxyl radicals (•OH's). The •OH react with DMSO to produce methane sulfinic acid and a methyl radical that in turn produces methane (Fig. 4.2).

Figure 4.2 DMSO scavenging of •OH



It is clear that careful interpretation of ethylene levels detected by GC is required, since ethylene biosynthesis and action requires intact membranes; a lower level of ethylene production may also indicate membrane damage, while an increase may indicate membrane recovery. Ethylene generation may be linked to another factor considered to be of importance in *in vitro* plant recalcitrance and growth retardation specifically the epigenetic changes, modulated by DNA methylation, through the common metabolic and biosynthetic precursor S-adenosylmethionine (SAM).

4.1.4.3 DNA methylation

During DNA methylation a methyl group from SAM is transferred to specific short DNA nucleotide sequences and is catalyzed by one of the DNA methyltransferase enzymes. Too little or too much methylation can inactivate or activate genes causing developmental and morphological irregularities.

Since these epigenetic modifications are susceptible to change, DNA methylation represents an excellent target to explain how environmental factors such as *in vitro* stress and manipulation may contribute to *in vitro* recalcitrance. It is postulated that the growth habit differences observed in this study between UAD and NRS cultures may represent epigenetic changes that may be detected by studying DNA methylation pattern changes.

Harding, *et al.*, (1996) first pioneered a method specific to tissue culture to monitor epigenetic changes in the form of rDNA methylation. This approach was proposed as an underlying mechanism of *in vitro* recalcitrance and growth retardation in mature shoot-tip cultures of *Vitis vinifera*. In DNA methylation, a methyl group from SAM is transferred to deoxyribonucleosides, with deoxycytidine as the main methylation site. DNA methylations are normally very tightly controlled via methylatransferases. Thus manifestation of changes in methylation pattern during growth and development (e.g. during *in vitro* ageing, vernalisation or stress) and through genetically controlled processes (Finnegan, *et al.*, 1995, Finnegan, *et al.*, 2000), may provide an insight into recalcitrance factors.

DNA methylation, in plants is a significant and widespread phenomenon, in some species up to 30% of total cytosine can be methylated (Adams and Burdon, 1985). DNA methylation, alters the binding of transcriptional factors, and therefore it can affect gene expression, which may be indicated by alterations in protein synthesis and therefore possibly detected by total protein analysis. The link between free radical production and DNA methylation has not been conclusively studied. It may be postulated that biochemical signals, such as $Ca,^{2+}$ plant hormones and reactive oxygen species, acting through signal transduction cascades activate genomic re-programming mechanisms that change cell metabolic state and defence systems (Joyce, *et al.*, 2003).

Reduced DNA methylation disrupts normal plant development. For example, *Arabidopsis* with reduced DNA methylation levels displayed a number of abnormalities including reduced stature, altered leaf size and shape, reduced root length and fertility and loss of apical dominance (Finnegan, *et al.*, 1995). Abnormal phenotypes became more severely manifested in successive generations of progeny from self-pollinated plants. In four to five generations of selfing plants with reduced methylation, >70% became infertile.

DNA methylation has been studied in *in vitro* plants to determine critical methodology factors (Johnston, *et al.*, 2006 In press) and in somaclonal variation, ageing and totipotency of *in vitro* woody plant cultures (Harding, *et al*, 1996). Restriction enzymes, determined that the methylation status of *V. vinifera* was altered, as a result of *in vitro* manipulations. Profiles of ribosomal DNA showed that the percentage of recognition sequences containing a methylated external cytosine increased from 7.7% in glasshouse-grown plants; to 64.5% in the first *in vitro* culture; to 72.5% following 1 year of *in vitro* growth.

A number of studies have demonstrated that under-methylated genes are associated with active expression, and highly methylated genes are considered to be silent (Klaas and

Amasino, 1989). Harding, *et el.*, (1996) using methylation sensitive restriction enzymes showed that growth of potato shoot-tips on mannitol-supplemented medium was correlated with subsequent DNA hypermethylation and this may be an adaptive response to high osmotic stress. The biological significance of the slow growth 'dormancy' may have been to conserve cellular resources.

HPLC analysis can be utilised to profile the smaller molecular weight fractions (first to be elucidated from the column) consisting of undermethylated nucleosides followed by larger methylated nucleoside fractions (DNA extracts elucidated later from the column). A HPLC method will be applied to Sitka spruce shoot-tip cultures to determine the global methylation status. This is the first report of an *in vitro* conifer DNA methylation investigation, but methylation status has been proven as a useful tool in other woody species, such as *Vitis vinifera* (Harding, *et al.,* 1996) particularly pertaining to post-cryopreservation damage and stability assessments.

4.1.5 Recalcitrance and impacts on cryopreservation

Cryopreservation has been achieved for *in vitro* shoot-tip apices of temperate tree species such as *Betula pendula,* (Ryynanen, 1998, Ryynanen and Haggman, 2001), *Malus* (Wu, *et al.,* 2001) and *Pyrus* spp.., (Reed, 1990) (Table 1.2). This has not been achieved for evergreen conifer tree species. Partly dormant *in vivo* bud explants were successfully recovered from -80°C storage (Kuoksa and Hohtola, 1991). Table 1.2 summarised the methodology used to cryopreserve shoot-tips from woody plant species.

Encapsulation-based methodology is particularly favourable to the contemporary large-scale genebankers, where automation may be developed to reduce specialist labour costs. The encapsulation-vitrification protocol, developed by Matsumoto, *et al.*, (1995), shows much promise for previously cryo-recalcitrant woody plant shoot-tip cultures and its application in this study will be the first report on a conifer species.

Recent advances in shoot-tip culture cryopreservation success are is in part due to vitrification protocols and improved cooling methodologies assisted by thermal analysis technology (Chapter 5) The wide range of pre– and post– cryopreservation treatments that assist in the osmotic and biochemical pre-conditioning and recovery of the shoot-tips (Benson, *et al.*, 2006 In press), are also beneficial. Treatments include a range of pre– and post– culture medium supplements such as sugars, sugar alcohols, amino acids and plant growth regulators often used in combination with modified photoperiod and temperature parameters. The first cryopreservation pre-treatment targeted in this project was to manipulate the freeze tolerant

mechanisms that fully cold-acclimated *in vivo P. sitchensis* buds utilise in the natural environment, specifically, the adaptive mechanisms that allow them to survive temperatures as low as -50°C throughout the winter.

4.1.6 Cryopreservation systems for woody plant shoot-tip cultures

4.1.6.1 Pre-treatments: harnessing cold acclimation and dormancy

Woody perennial plant ecophysiology studies can apply frost tolerance adaptations to help develop cold acclimation pre-treatments prior to cryopreservation. Characteristics of full winter dormancy include the formation of resting buds, suspended growth, a decrease in metabolic activity, adaptations to resist desiccation and structural and cytoplasmic cellular level changes (Havranek and Tranquillini, 1995). To avoid intracellular freezing, water in the cells must be either capable of supercooling or moved to extracellular spaces. Intensive changes also occur at the cellular level including alterations in sugar, amino acids, nucleic acids, proteins, lipids, abscisic acid, and cytological structures.

Cold hardy conifers have evolved from four northern genera of *Pinaceae: Pinus, Picea, Larix* and *Abies* (Sakai, 1983). Extraorgan freezing is employed as a survival mechanism for the shoot and flower primordia of all genera except, *Pinus*. Extraorgan freezing is defined as ice segregation outside an organ so that an organ may survive freeze-induced dehydration, preventing ice penetration into the organ. In conifers belonging to the subgenus Abietoideae (*Picea, Abies, Larix*), the mechanism of limiting ice formation to the crown of the shoot controls the movement of water from the primordia allowing the primordia to survive to -40°C or lower. The primordia of *P. glauca* and *P. mariana* have been shown to survive up to -70°C.

To achieve cold hardening in nature, subfreezing temperatures (-5 to -10 °C) are required and have been correlated with large invaginations of the chloroplast envelope, an increase in the number of chloroplasts and mitochondria, the migration and conglomeration of chloroplasts in the cell and the dispersion of large vacuoles into numerous smaller ones (Havranek and Tranquillini, 1995).

Dormant buds sampled from trees in field collections have been successfully cryopreserved for several woody plant including *Pinus* (Kuoksa and Hohtola, 1991) and *Malus* spp. (Wu, *et al.*, 2001). In *Malus*, the recovering explants showed low recovery *in vitro* (16%) associated with high contamination (up to 50%). Cold acclimation or hardening of *in vitro* grown shoot-tips has been utilised for *Rubus* (Reed, 1988), *Vitis* (Zhao, *et al.*, 2001) and *Pyrus* spp. (Chang

and Reed, 2000) meristems (Table 1.2). Cold acclimation or hardening was induced in different ways. A system incorporating shoot-tips at a constant low temperature of 4 to 5°C in total darkness has been used for the cryopreservation of *Malus* sp. (Chang, *et al.*, 1992).

Chang and Reed, (2000), incorporated a system of 'alternating-temperature,' whereby *in vitro Pyrus* shoot-tip meristems were maintained (at 22°C with light for 8hr and 1°C in darkness for 16hr) for 1 to 15 weeks. Alternating-temperature shoot acclimation for 2-5 weeks significantly increased post-cryogenic meristem re-growth. In *Vitis vinifera* (Zhao, *et al.*, 2001), buds destined for cryopreservation were sampled from mother-plants that had been kept without sub-culture for 3 to 4 months with a one-month period of alternating cold acclimation in 8hr light/16hr dark at 5°C.

Researchers have also incorporated pre-treatment combinations of sugars and plant growth regulators with cold acclimation regimes. It is thought that these pre-culture additives assist in priming the explant for cryopreservation by inducng the beneficial physiological changes of cold acclimation and by dehydrating tissues and stabilizing proteins and membranes during desiccation (Chang and Reed, 2000). *In vitro* shoot-tips of *Betula pendula* were cold hardened for 28 days at 5°C under an 8/16hr light/dark photoperiod on medium containing 10^{-4}M ABA resulting in a post-cryopreservation shoot recovery of over 40% (Ryynanen, 1998).

Optimised shoot pre-culture regimes of 3 wk culture on 5-7% (w/v) sucrose medium followed by 2 weeks low temperature (4°C) were shown to increase post–cryopreservation shoot-tip growth from 0-70% in *Pyrus cordata* (Chang and Reed, 2001). The physiological investigation of a number of pre-culture additives in combination with cold acclimation remains to be undertaken. The amino acid proline (10% w/v) was incorporated successfully as a pre-growth additive in the freeze preservation of *Zea mays* (Withers and King, 1979). It is favoured for investigation due to its proven properties in desiccation stress resistance in higher plants and because it has a high solubility, is pH neutral and non-toxic at high concentrations.

Trehalose has been used successfully as a pre-culture additive for the cryopreservation of *Digitalis lanata* cell cultures (Goldner, *et al.*, 1991) where high post-thaw viability rates were achieved with only a short lag period. Trehalose occurs naturally in a large range of desiccation and freeze tolerant organisms ranging from bacteria to invertebrate animals (Wingler, 2002). Trehalose has been reported to show special properties relating to cell membrane stabilisation (Turner, *et al.*, 2001a) and glass stability during freezing (Benson., *et*

al., 2005 In press, Wang and Haymet, 1998). Interestingly, trehalose is produced by mycorrhizal root fungus in winter, correlating positively with frost and desiccation tolerance in *P. abies* (Niederer, *et al.*, 1992). There are no reports of trehalose being used for cryopreservation pre-culture in conifer shoot-tips or in combination with cold acclimation but it is thought that its special properties and ecophysiological association with conifers may greatly assist in the cyopreservation of *P. sitchensis in vitro* shoot-tips.

4.1.6.2 Pre-treatment: wounding and dissection injury

In preparation for Na-alginate encapsulation and cryopreservation it is necessary to dissect the shoot meristem or apex from the protective shoot-tip. This is a delicate procedure and sometimes dissection injury can result. To ameliorate the effects of wounding, following dissection the meristem may be incubated on a filter bridge soaked with basal medium (Withers, *et al.*, 1988) and DMSO (Benson, *et al.*, 2006 In press). The exact repair properties of DMSO are unknown but it has free radical scavenging capabilities and has been shown to induce cellular differentiation and function in leukaemic mammalian cells (Jacob and Herscheler, 1986).

4.1.7 Post–cryopreservation culture

The development of an optimal recovery and regeneration medium is critical for the improvement of the post–cryopreservation recovery of shoot-tips. In many species it has been observed that plantlets initially showing high post cryopreservation survival proceed to senesce in the following 2-4 weeks because of sub-optimal recovery conditions (Turner, *et al.*, 2001b). Also it is probable that if a cold hardening treatment is applied before cryopreservation, there will be a degree of dormancy and a treatment may be required to re-initiate active growth. In natural stands of forest trees, post-dormancy physiological activity is gradually restored under the influence of phytohormones (Lavender and Silim, 1987). For example, the recovery of *Anigozanthus viridis* shoots was improved through culture in a post-cryopreservation medium containing a combination of 0.5M cytokinin (kinetin or zeatin) and 0.5M GA_3 (Turner, *et al.*, 2001b). Cytokinins stimulate cell division and reduce apical dominance while GA_3 triggers internode elongation. Thiadiazuron has also been reported to stimulate shoot proliferation in woody plant species and it is thought to induce axillary shoot proliferation in concentrations < 1μM (Huetteman and Preece, 1993). To progress the physiological understanding of storage recalcitrance in Sitka spruce shoot cultures, all the aforementioned parameters will be explored experimentally.

4.3 Materials and Methods

4.2.1 Experimental rationale and design

A programme of physiological investigation and experimentation was devised to assist the current understanding of problems associated with the technical and practical incorporation of cryopreservation to Sitka spruce shoot cultures. Table 4.1 provides a summary of the approach.

Table 4.1 Rationale summary for applying physiological approaches to Sitka spruce shoot culture cryopreservation

(1) Stage 1 *In vitro* standardisation

Rationale	Experimental parameter	Justification	Contribution
Assurance of growth parity between different partner institutes	(1) Accumulative shoot extension (cm) (2) Vigour/morphology	Standardisation of culture conditions	Determines if growth room parameters impact storage behaviour
Genotypic differences	(1) Accumulative shoot extension (cm)	Identification of genotypes for cryopreservation	Indicates genotype variability and requirements for pre-cryogenic treatments
Biochemical assessment of location and genotypic differences	(1) Proteins and antioxidants (2) DNA methylation	Identification of physiological markers	Allows physiological and biochemical comparisons

(2) Stage 2 Cryopreservation pre-culture and pre-treatment developments

Rationale	Experimental parameter	Justification	Contribution
Identification of cold acclimation	1-6 weeks cold hardening regime	Optimise hardening/acclimation based on tolerance responses	Improvement of tolerance to cryogenic pre-culture
Cold hardening and biochemical additives	Cold hardening and chemical additives (sucrose)	Chemical additives (sucrose, trehalose, proline and ABA) shown to assist in desiccation and freezing tolerance.	Enhancement of cryopreservation
Enabling recovery from dissection damage	1-5% (w/v) DMSO application in filter bridge medium 24hr	DMSO acts an •OH scavenger	Enhances survival

(3) Stage 3 - Cryopreservation techniques

Rationale	Experimental parameter	Justification	Contribution
Encapsulation –dehydration cryopreservation applied to characterise critical growth factors	Explant source location, size and developmental status	Optimisation of key parameters	Determination of optimal cryopreservation explantusing informed physiological parameters
Encapsulation-dehydration protocol desiccation time strategies	Osmotic dehydration and desiccation tolerance + RM studies	Identification of desiccation limits	Optimisation of pre-cryogenic desiccation
Encapsulation-vitrification	Optimise PVS2 vitrification	Identification of toxicity limits	Comparison of cryopreservation protocols

(4) Stage 4 - Post cryopreservation recovery

Rationale	Experimental parameter	Justification	Contribution
Enhancement of recovery	GA_3, TDZ and BAP	Stimulation of meristem growth	Enhancement of plant regeneration following cryopreservation
Maintain shoot-tip apice in bead or dissect out following treatment	Compare survival and re-growth rates in each case	Optimise post-treatment recovery	Optimal plant regeneration following cryopreservation

4.2.2 Routine culture

Routine culture

Twenty-two clones of shoot cultures from five specific cross (full sibling) families (designated A- E) were transferred from Northern Research Station (NRS) to the University of Abertay Dundee. Shoot-tips were cultured at 20°C with a photoperiod of 16hr a day. At NRS the light regime comprised of cool white fluorescent bulbs (Thorn Natural Weiss Deluxe, 125 Watt.), 50 μ mol m^{-2}/s and at UAD, cool white fluorescent bulbs (Osram Sylvania) 50 to 70μ mol m^{-2}/s. Shoot cultures were maintained in soda glass tubes [(Oxoid^{TM}) 100mm (height) x 25mm (diam.)] containing 20ml of MS medium (Murashige and Skoog, 1962). Temperatures were ascertained using a probe placed in the soda tubes. Shoot tips were sub-cultured every 8-10 weeks dependent on their growth rate and development.

Qualitative assessments and quantitative measurements

Qualitative assessments of shoot-tip growth pattern and colour were undertaken, whilst quantitative measurements of apical shoot growth were performed. Growth was measured *in situ* as total accumulative extension (cms) from the base of the shoot at the agar /air interface to the top of the primary shoot apex. Qualitative and quantitative comparisons were made between: genotypes at UAD and genotype AC cultured for 6 months at UAD or NRS.

Comparisons between culture locations were recorded for genotype AC over 42 days (NRS) and 49 days (UAD). Growth (cm) was measured at 7-day intervals and expressed as a percentage of the original subculture length to account for minor differences in subculture practices at UAD and NRS. Growth (cm) comparisons between genotypes of five families at UAD were recorded over 32 days. In each case, ten replicate shoot-tip cultures were used to determine the mean $\pm$ SEM.

4.2.3 Biochemical markers

Biochemical markers were used to assess differences between genotypes in culture at UAD. Total aqueous protein and total antioxidant activity assays were performed on genotypes AA and AC. Biochemical markers were also applied to assess the differences between equivalent genotypes cultured for 6 months at different locations.
Ethylene evolution was determined using GC headspace analysis applied to genotype AC shoot cultures from UAD and NRS. DNA methylation analysis was undertaken on genotype AA cultures from UAD and NRS.

4.2.3.1 Tissue extraction

Pre-weighed (1g) Sitka spruce *in vitro* shoots were transferred to a pre-cooled ceramic mortar filled with LN, and were ground with a pestle to a fine powder. A LN–cooled metal spatula was used to transfer the tissue (50-100mg) from the mortar to LN cooled 1.5ml microcentrifuge tubes. Samples were removed from LN and 0.5ml of cold (4°C) extraction buffer added.

Extraction buffer consisted of 50mM KH_2PO_4 with 1mM $CaCl_2$, 1mM KCl and 1mM EDTA, adjusted to pH 7.0 with 50mM K_2HPO_4 containing the same salts (Murphy and Huerta, 1990). Buffer was added to the samples and both were homogenised for 30 sec using mini-pestles (Sigma), and incubated on ice for 20min, vortexing every 5 min. The extract was centrifuged (Jouan SA, A14, St. Herblain) at a relative centrifugal force of 12,300 *g* for 5-10 min at 4°C. The supernatant was transferred to microcentrifuge tubes on ice and the pellet was discarded. Assays for total aqueous antioxidants were performed without delay, and the remaining extract stored at -20°C until required for total protein analysis.

4.2.3.2 Total water soluble protein determination

Total soluble protein activity was determined spectroscopically using Coomassie protein binding dye (Bradford, 1976). Coomassie Brilliant Blue G-250 (Sigma) (100mg) was dissolved in 50ml ethanol (95% v/v) and 100ml phosphoric acid (85% w/v). The volume of reagent was made up to 1000ml with deionised H_2O, and stored in the dark at ambient temperature. Protein standards were prepared by dissolving bovine serum albumin (BSA FractionV, Sigma) in phosphate extraction buffer at concentrations of 0-0.5mg/ml.

A vortexed sample/standard of 0.05ml was added to 1ml of reagent in a 1.5ml plastic cuvette, covered with parafilm and inverted three times to mix. The mixture was incubated at

ambient temperature for 2min, and read in a spectrophotometer (Cecil Series 2 CE292) at an absorbance of 595nm within 15 min. Samples and standards were performed in triplicate and the protein concentration determined using a standard curve and results expressed on a fresh tissue weight basis using the following equation:

$$\text{Total protein (mg/gFW)} = \frac{\text{protein conc (mg/ml) x extract vol (ml)}}{\text{extracted tissue weight (g)}}$$

4.2.3.3 Total aqueous antioxidant activity

Total antioxidant activity was determined by measuring the depletion of azino-3-ethylbenzothiazoline-6-sulfonic acid radical chromophore (Johnstone, *et al.*, 2003, Johnstone, *et al.*, 2005 In press, Re, *et al.*, 1999). Activity was expressed as trolox equivalents. A radical stock solution was prepared by adding 0.0960g of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid diammonium salt (ABTS; Sigma) to 25ml of deionised H_2O to give a final concentration of 7mM. Potassium persulfate (0.0166g) (Sigma) was added to the ABTS solution giving a final potassium persulfate concentration of 2.45mM. The solution was stored in the dark at 20°C for 12-16hr prior to use.

The spectrophotometer was blanked at 734nm using a cuvette (1.5ml plastic) containing 1 ml of phosphate buffered saline. Radical stock solution was diluted with 5mM phosphate buffered saline (pH 7.4) until the spectrophotometric absorbance value of 0.7 to 0.73 at 734nm was obtained. Phosphate buffer saline consisted of 5mM NaH_2PO_4 /37.5mM NaCl in deionised H_2O and was adjusted to pH 7.4 using 5mM Na_2HPO_4/37.5mM NaCl in deionised H_2O. In all assays, 10µl of sample/standard was added to 1ml of dilute radical solution (A_{734}nm =0.7-0.73) and absorbance reading taken at 30sec, 1 and 2 min after initial mixing (covered with parafilm and inverted 4 times). A standard curve of total antioxidant activity in 0-1500µM trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) standards made up in phosphate extraction buffer was produced and used to determine antioxidant activity in samples/standards. The results were expressed per g of fresh weight extracted tissue and per mg protein. Each assay was performed in triplicate for each replicate sample or standard.

4.2.3.4 Volatile hydrocarbons headspace analysis

Glass vials (16ml $\pm$ 0.02ml) were loosely sealed with open-top caps and inert silicon/teflon septa (Supleco) and autoclaved for 20 min at 121°C/10bar in the absence of plastic. In order to avoid volatile contamination after autoclaving each vial was immediately vented in a laminar flow bench for 1-2hr. MS medium (10ml) was added to each vial using a 5ml pipette,

and vials were placed on tilted racks (ca 45° angle) to maximise the surface area of the medium. Following medium solidification the vial was further vented for 10 min.

DMSO [5% (v/v)] was added (200µl) to each vial. Shoot-tip tissue fresh weight, between 50-100mg pooled from 1-5 shoot-tips, was recorded and transferred to the vial, which was sealed immediately with a record of the time and weight. Control vials without plant tissue, were incorporated to determine background volatiles (derived from DMSO, media and laboratory atmosphere). The headspace volume was calculated using the following equation:

Headspace vol. = vial vol.-(medium vol.+ DMSO vol.+ plant tissue vol.)

Vial headspace composition was analysed by gas chromatography for volatile hydrocarbons approximately 41hr after sealing. Gas chromatography of headspace samples (1ml) was performed using a Cambridge Scientific Instrument 200 series gas chromotograph fitted with a Heysep Q packed column (2m long, 1/8"ID stainless steel tubing, 80-100 mesh) and a hydrogen/air flame ionisation detector at 225°C linked to a computer equipped with integration software (GC data station vs 3 CSW32, Windows 2000). An isothermal column oven temperature of 60°C and an analysis time of 6 min was used to separate volatiles. Oxygen –free nitrogen was used as the carrier gas and set at a flow-rate of 25 to 30ml/min. Volatile hydrocarbon chromatographic peaks of methane and ethylene were identified using gas calibration standards (Scotty I analysed gases, product number 2-2566). Standards consisted of ethylene 30ppm, ethane 15ppm, methane 15ppm. Headspace samples (1ml) were drawn from vials, using a 1ml gas-tight syringe and were flushed several times to mix headspace before removal and injection into the gas chromatogram column. Three injections per replicate (vial) were undertaken.

Volatile production was calculated using the following equation:

$$r_{volatile}\,(mol/s/g) = \frac{(sconc \times sh/vol) - (bconc. \times bhvol.)}{\frac{(22.4 \times temp. \times 10^6)}{273.15}} \times \frac{1}{t_2 - t_1} \times \frac{1}{FW}$$

Where:

$r_{volatile}$=rate of volatile production

sconc.= sample concentration in 1ml sample (µl/l)

bconc.=blank concentration in 1 ml sample (µl/l)

sh.vol.= sample headspace volume (l)

bhvol.= blank headspace volume (l)

t_2-t_1=time between sealing and removal of 1ml sample

FW=sample tissue fresh weight (g)

temp.=temperature during sampling (K)

22.4=l of space occupied by 1 mol of gas at 273.15K and 1 atm.

10^6=conversion factor from l to µl

4.2.3.5 DNA methylation HPLC analysis

(a) DNA extraction

A method using cetryltrimethyl ammonium bromide (CTAB) was used to extract DNA from Sitka spruce *in vitro* shoot tips (Lodhi *et al.,* 1994). Stock solutions consisted of: Tris – Annals of -aminomethane buffer (Tris), 1M Tris base in deionised H_2O pH8; 0.5M ethylene diamine tetra-acetic acid (EDTA) in deionised H_2O pH 8; and 5M NaCl in de-ionised H_2O. CTAB extraction buffer was prepared with 50ml of 1M Tris, 20ml 0.5M EDTA, 140ml of 5M NaCl, 10g CTAB and adjusted to pH 8 made to 500ml with deionised H_2O.

Polyvinylpyrrolidone (100mg per g of tissue) and dithiothreitol (final concentration of 10mM) was added to an aliquot of stock CTAB extraction buffer to produce a working CTAB extraction buffer on the day of extraction. The CTAB extraction buffer was pre-heated to 65°C for 1hr. LN pre-ground tissue (50-100mg) was transferred to LN-cooled, 1.5ml microcentrifuge tubes, and 0.5ml of pre-heated extraction buffer added. Samples were thoroughly mixed by gentle inversion and incubated at 65°C for 20min and mixed every 10 min. Samples were held at room temperature for 3min and 0.5ml 4% (v/v) isoamyl alcohol/chloroform added, the tubes were gently inverted 20 times and centrifuged at a relative centrifugal force of 6000 *g* for 15min. The upper aqueous layer was transferred to clean 2ml microcentrifuge tubes and the interphase and the organic phases discarded. NaCl (0.25ml 5M) was added, the tubes inverted 20 times to mix and 1ml of cold (-20°C) 95% (v/v) ethanol was added. Samples were inverted to mix and incubated at 4°C for 30min.

The extract was centrifuged at 5000 *g* for 6 min. The supernatant was discarded and the pellet washed in 200µl of cold (–20°C) 70% (v/v) ethanol. The pellet was re-centrifuged at 5000 *g* for 5 min, the supernatant removed and the pellet allowed to dry at room temperature. The pellet was re-suspended in 100µl of deionised H_2O and stored at -20°C until required for digestion.

(b) DNA digestion method

The concentration of DNA in each sample was determined using spectrophotometric methodology and the following formula:

DNA Conc. (µg/ µl)=A_{260}nm x 50µg/ml x DF $\frac{760}{10}$ x CF $\frac{1}{1000}$

Where:

A_{260}nm= Absorbance at 260nm of 10µl of suspended DNA in 750µl of deionised H_2O in a quartz cuvette.

DF= Dilution factor µl deionised water/ µl of sample volume

CF= Conversion factor to change units to µg/ µl

Suspended DNA (25µg) was made to a final volume of 100µl of deionised H_2O in a microcentrifuge tube. A sample containing 100µl of deionised H_2O and no extract was incorporated as digest blank. The samples were boiled for 2 min and flash cooled to produce single strands of nucleic acid for digestion. A 5 µl aliquot of 10mM $ZnSO_4$ and 10µl of 1U/ml nuclease P1 (from *Penicillium citricum,* Sigma) was added to each sample which was gently mixed and incubated at 37°C for 17hr. An aliquot of Tris (10 µl, 0.5M pH 8.3) followed by 10 µl 5 U/ml of bacterial alkaline phosphatase (from *Escherichi coli,* Sigma) was added and the mixture incubated at 37°C for 2hr. Samples were centrifuged at 10,000 *g* and the supernatant transferred to clean microcentrifuge tubes. Samples were stored at -20°C until HPLC analysis.

4.2.3.6 HPLC analysis of nucleosides

A HPLC unit consisting of a gradient pump (Spectra Physics SP880 ternary HPLC pump), a 20µl injection loop, a Synergi-max 4µM C12 column, 250mm x 4.6mm, a guard column (SecurityGuard 4 x 3mm cartridge), a UV detector (Severn Analytical SA 6500) and an integrator (Spectra Physics, Chrome jet indicator) was used to assess the methylation status of samples and standards (Tables 4.2 and 4.3). Four de-gassed solution buffers [90% (v/v) methanol; 10mM phosphate buffer in 0.5% (v/v), pH 3.75; 10mM phosphate buffer in 10% (v/v) methanol pH 3.75; deionised H_2O] were incorporated in a gradient programme to prepare the HPLC column (Table 4.4). A 50-µl injection syringe was flushed with deionised H_2O between samples as was the injection loop.

Table 4.2 Nucleoside standard calibration

Standards	RNA (μM)	DNA (μM)	5-M-dc/5-M-c*(mM)
1	15	5	1.5
2	75	25	7.5
3	150	50	15

Each sample was injected once and 3 replicates were performed in each genotype and location.

*5-M-dc= 5 methyl deoxycytidine; 5-M-c= 5 methyl cytidine

Table 4.3 Nucleoside Standards

Nucleoside	Abbreviation	Standard Range (μl)
2'-deoycytidine	DC	5-50
2'-cytidine	C	15-150
5-methyl–2'-deoxycytidine	5-M-dC	1.5-15
5-methyl-2'-cytidine	5-M-C	1.5-15
2'-thymidine	T	5-50
2'-uridine	U	15-150
2'deoxyguanosine	DG	5-50
2'-guanosine	G	15-150
2'-deoxyadenosine	DA	5-50
2'-adenosine	A	15-150

Table 4.4 HPLC gradient programme for running standards* and samples

Time (min)	Solution A- 90% (v/v) methanol	Solution B- phosphate buffer in 0.5% (v/v) methanol	Solution C- phosphate buffer in 10% (v/v) methanol	Flow rate (ml/min)
0	0%	100%	0%	1.00
10	0%	0%	100%	1.00
25	0%	0%	100%	1.00
26	0%	100%	0%	1.00
30	0%	100%	0%	1.00

*Integrator calibrated daily using these standards

4.2.4 Pre-culture optimisation

4.2.4.1 Shoot culture cold hardening

Vigorous shoot-tips (Family A) selected 4 weeks after sub-culturing were dissected to 2cm and transferred to compartments in a sterile 100mm sq. Petri dish (Bibby Sterilin, UK, Cat. Nos. 103) that were filled with solid MS medium (1ml). Each square Petri dish consisted of 25 compartments and shoot-tip replicates undergoing the same pre-culture regime were placed in the same Petri dish. Each set of shoot-tips were cold pre-cultured for 1 to 6 weeks: 1 week under continuous 4°C (in the dark) or for 2-6 weeks under alternating 8hr 20°C and cool white fluorescent light (Osram Sylvania) 50 $\mu mol/m^{-2}/s$ and a 16hr at 4°C (in dark).

Following pre-culture the shoot-tips were transferred to soda tubes and fresh MS medium and were cultured under routine light and temperature (4.2.3). The % survival and the re-growth in cm was recorded following cold acclimation. The mean $\pm$ SEM % survival was determined from the number of green shoot-tips, from five replicate shoot-tips from four replicate experiments (n=20). Mean $\pm$ SEM % re-growth (cm) was determined from the accumulative growth of the leading shoot-tip apices in 5 replicate shoot-tips.

4.2.4.2 Controlled rate cooling of cold hardened (sucrose) shoot-tips

In vitro shoot-tip apices were cold hardened for 1 week in either MS medium or MS medium supplemented with 5-11% (w/v) sucrose, added to simulate cold-hardening. Cold hardened

and non-hardened control shoot-tips were dissected to 2cm in length (including apex). Cryovials (Simport ™, self standing) containing moist (with MS liquid) sterile tissue paper placed on ice were prepared. Five shoot-tips were transferred to each cryovial, which were transferred to cryo-straws and placed in a Programmable Freezer (Planar, UK, KRYO 10 Series 111). A program from 0 to -25°C was initiated at rate of -0.1 °C /min with -5°C holds where 2 cryovials (10 replicate shoot-tips) were removed. The program continued with -5°C increments and subsequent removal of cryovials, until a terminal temperature of -25°C was attained. A temperature probe linked to a computer processing unit and a printer was placed in a cryovial containing sterile tissue moistened with liquid MS media. Cryovials were removed from the Programmable Freezer and placed in a chilling cabinet (6°C) for 2hr. The cryovials were washed with Hibitane,™ transferred to a laminar-flow bench, shoot-tips were removed and cultured on solid MS medium in soda tubes. Shoot-tips were assessed weekly for 6 weeks and those that were green were recorded as surviving. Five replicate shoot-tips from 2 cryovials were assessed per treatment and the number of surviving shoot-tips from each set of five was recorded to provide a mean ± SEM.

4.2.4.3 Shoot-tip pre-culture additives

Vigorous shoot-tips (Family A) that had been sub-cultured 4 weeks previously were cultured for 1 week under continuous 4°C dark conditions on solid MS medium supplemented with pre-culture additives:

(a) 5-11% (w/v) sucrose
(b) 5-11% (w/v) trehalose
(c) 5% (w/v) proline
(d) 10^{-4} – 10^{-5} M ABA – filter sterilised and added to medium following autoclaving (dissolved in 100% ethanol)

Pre-cultured shoot-tips were encapsulated, osmotically dehydrated, desiccated in a laminar flow bench and immersed in LN. The shoot-tips were re-warmed, re-cultured and assessment undertaken following control and post-LN cultures. For each pre-culture supplement, five replicate shoot-tips were assessed for survival for each experiment, which was undertaken twice. The mean shoot-tip survival ± the SEM was determined.

4.2.4.4 DMSO pre-treatment

DMSO aliquots of 0, 1, 3 and 5% (v/v) were asceptically prepared in liquid MS medium and applied to sterile tissue paper in Petri dishes. Shoot-tips (Family A) sub-cultured for four weeks previously, were dissected to an optimised size, placed in sterile Petri dishes (containing one of the 0-5% (v/v) DMSO treatments), sealed with clingfilm and placed in the dark at 4 °C for 24hr. Shoot-tips were recovered on solid MS media with medium changes on days 2 and 4. Shoot-tips were assessed for survival (without callus formation) on days 1,7, 14 and 28. Five replicate shoot-tips were assessed per experiment and the experiment was undertaken twice. The mean ± SEM was determined from the two experiments.

4.2.5 Encapsulation-dehydration

4.2.5.1 Shoot-tip meristem excision

Shoot-tips from family A were dissected to three sizes as shown in Figure 4.3. Size 1 (0.5 -1 mm) no leaf primordia included, size 2 (1-2 mm) included apical primordia, with emerging scales and crown, and size 3 (2-3 mm) included apical or axial core pith.

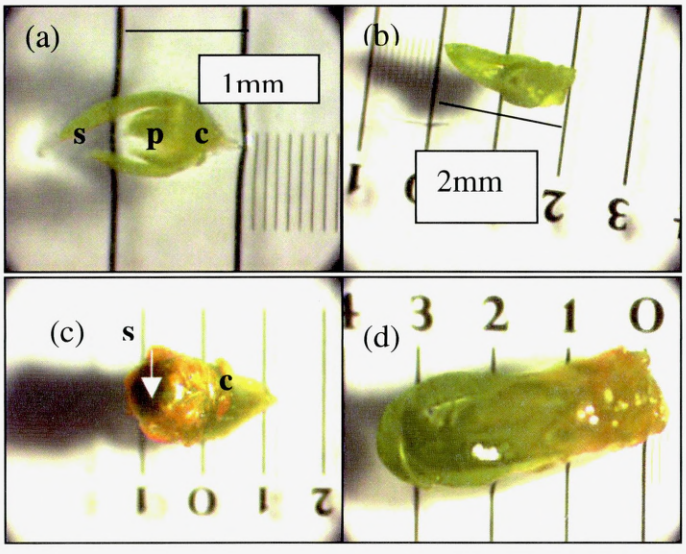


Figure 4.3 Excised morphology of shoot-tips

(a) Size 2 apical shoot-tip apex, (b) Size 3 apical shoot-tip apex, (c) Size 1 lateral, dormant bud, (d) Size 3 lateral bud breaking out of dormancy; p= primordia; c= crown; s= scales (bud)

Meristems were excised from the apex of the shoot-tip and from dormant lateral /axillary branching buds (Fig. 4.4).

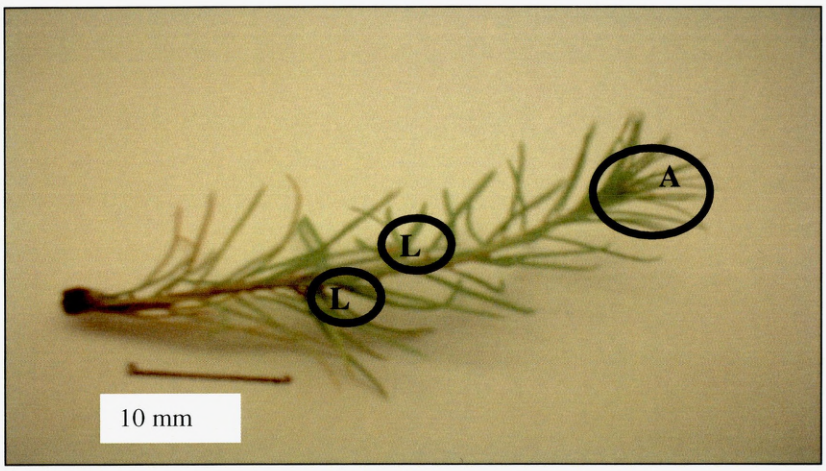


Figure 4.4 Locations of dissection: A =Apical ; L= Lateral

4.2.5.2 Encapsulation□dehydration

Freshly dissected meristems were placed in a 3% (v/v) Na-alginate solution and 3 ml was dispensed with several meristems using a 5ml pipette. The mixture was dropped into calcium liquid medium. On contact with Ca^{2+} the droplets solidified, encapsulating the shoot tips. The beads were left to polymerise for 20 min. Osmotic dehydration of encapsulated shoot-tip apices in 0.75M sucrose was undertaken by immersing the encapsulated shoot-tips in 0.75M (prepared in liquid MS) sucrose solution for 18hr on a rotary shaker. Laminar air-flow desiccation of encapsulated, osmotically dehydrated shoot-tip apices was undertaken for 3-4hr. Following desiccation, encapsulated apices were placed in a cryovial, slotted into a cryocane, with a cryocover and placed in a mini Dewar of liquid nitrogen for 20 minutes.

Following LN immersion the cryovials were transferred rapidly from the storage Dewar to a polystyrene float in a 40°C water bath for approximately 2 to 3 min. The vials were surface sterilised (Hibitane™), transferred to a flow bench and the beads emptied into sterile Petri dishes. Beads that had undergone osmotic dehydration and/or desiccation were re-hydrated in liquid MS media for 20 min. Three replicate shoot-tips per size/location/treatment were placed in individual 55mm diameter Petri dishes containing 15ml of solid MS media. Each replicate was cultured for 1 week before the viability (% green area) was assessed. Meristems were examined weekly for up to 6 weeks after treatment.

4.2.5.3 Dehydration and desiccation parameters

The % relative moisture content (% RM) was determined for (1) blank beads without sucrose osmoprotection, (2) blank beads with sucrose osmoprotection and (3) beads with a shoot-tip apex and osmoprotected. Fresh beads were weighed (FW) before and during laminar air flow desiccation at hourly intervals for 6 hr and were then placed over silica gel at 105°C oven for 12hr to determine the dry weights (DW). Mean % RM ± SEM content, for each hour of desiccation was determined from 10 replicate beads.

% RM = (FW – DW)/FW x 100

Shoot-tip survival following 0-6hr laminar air-flow desiccation was also determined for encapsulated shoot-tips that had or had not undergone cold hardening for 1 week. The mean ± SEM % of shoot-tip showing re-growth after 28 days was determined from two experiments each consisting of 10 replicate shoot-tips.

4.2.6 Encapsulation-vitrification

Following optimised pre-culture shoot-tips were cryopreserved using an encapsulated-vitrification protocol (Hirai and Sakai, 1999, Matsumoto, *et al.*, 1995, Sakai, *et al.*, 2000, Shibli and Al-Juboory, 2000) that incorporated an osmoprotection step of 2M glycerol and 0.4M sucrose followed by PVS2 treatment.

Pre-treated shoot-tip apices were encapsulated in 2% (w/v) Na-alginate beads containing 0.4M sucrose. Encapsulated shoot-tip apices were osmoprotected with 2M glycerol and 0.4M sucrose (prepared in liquid MS) for 1 hr on a rotary shaker at ± 20 °C followed by PVS2 incubation for 0, 30min, 1 and 2 hr at 0 °C (on ice). The beads were transferred to 2 ml cryovials (Simport™, self standing) and re-suspended in 1.8ml of fresh PVS2 (5 beads per cryovial). The cryovials were immersed in LN for at least 24hr and then re-warmed rapidly in a water bath at 45 °C (ca. 2min). The PVS2 solution was drained from the cryotubes and the beads were transferred to sterile Wilson sieves, 100µm pore size, and rinsed with 1.2M sucrose made up in liquid SEIM at pH 5.8. The beads were placed on sterile filter paper to drain excess PVS2 and placed on solid MS medium. Each experiment consisted of five replicate encapsulated shoot-tip apices per treatment, and experiments were undertaken twice. The results were pooled and displayed as means ± SEM.

4.2.7 Post-cryopreservation optimisation

Plant growth regulators

Shoot-tip apices were dissected and placed on solid MS medium supplemented with filter-sterilised GA_3, BAP or TDZ (0.5, 0.1 μM) and re-growth monitored after 28 days. Each assessment included the control of the hormone/PGR diluent. Sterile, 100mm square Petri dishes (Bibby Sterilin, UK, Cat. No. 103) containing 25 1ml volume compartments were filled with 1ml of warm (still liquid) MS solidifying medium and 10 μl of PGRs in diluents.

1)
   (a) 0.5μM GA_3 (100% ethanol)
   (b) 1μM GA_3 (100% ethanol)
   (c) control 100% ethanol

2)
   (a) 0.5μM TDZ (1M KOH)
   (b) 1μM TDZ (1MKOH),
   (c) control 1M KOH

3)
   (a) 0.5μM BAP (dissolved in 0.5M NaOH and distilled water)
   (b) 1μM BAP (dissolved in 0.5M NaOH and distilled water)
   (c) control 0.5M NaOH and distilled water

Untreated shoot-tip apices were dissected to 2cm and placed in the dish compartments, which were maintained under routine light and temperature for 14 days. The shoot-tips were subsequently transferred to MS medium without PGR supplements. At least 10 replicate shoot-tips were used for each treatment per experiment and each experiment was duplicated. Survival on the PGR treatment was assessed after 28 days as the number of green shoot-tips. Data from the two experiments was pooled and expressed as the mean ± SEM.

Bead removal after 3 days

Investigations were undertaken to compare the response of removing dissected meristems or buds after 3 days and monitoring the number of shoots that were alive after 48 days of culture with and without alginate bead removal. Each experiment consisted of 3 beads and the experiment was undertaken twice.

4.3 Results

4.3.1 Optimisation of pre-storage culture morphology and growth

Qualitative and quantitative growth assessments indicated a difference between *in vitro* shoot-tip culture growth at University of Abertay Dundee (UAD) and Northern Research Station (NRS). Figure 4.5 shows physiological differences between identical genotypes AC as stunted growth, yellowing and the apical bud was closed as in dormancy in the UAD cultured shoot-tips. Temperature probes inside the culture tubes indicated that initially there was a temperature difference in microenvironments; NRS (20°C±1°C), UAD (22-25°C). This difference was reduced at UAD by placing thermal matting under the soda tubes producing temperatures of 22-23°C.

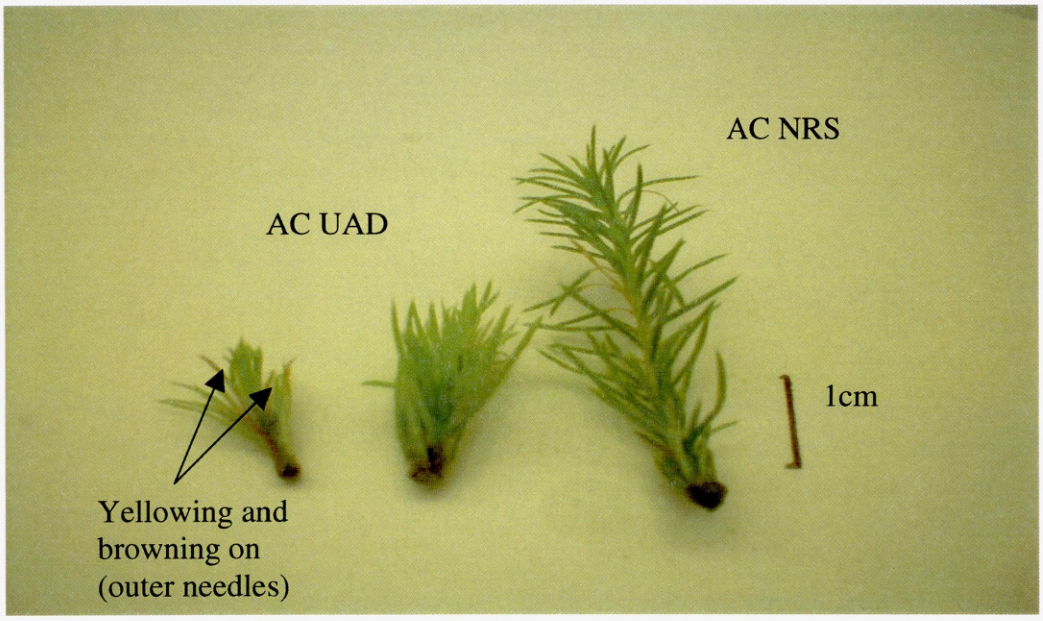


Figure 4.5 Images of shoot-tips of (genotype AC) maintained at UAD and NRS (University of Abertay) growth room and NRS (Northern Research Station) growth room cultures were assessed after 2 subculture cycles (= 6months)

The mean % accumulative growth (cm) in NRS shoot-tip cultures was ca. 105% greater than UAD equivalent cultures by day 42 (Fig.4.6) and UAD shoot-tip cultures did not increase after day 28. A comparison of the mean accumulative extension (cm) of shoot-tip genotypes (families A to E) cultured at UAD showed that the mean growth of family A after 28 days was double (0.8cm) that of any of the other families, (see Fig. 4.7).

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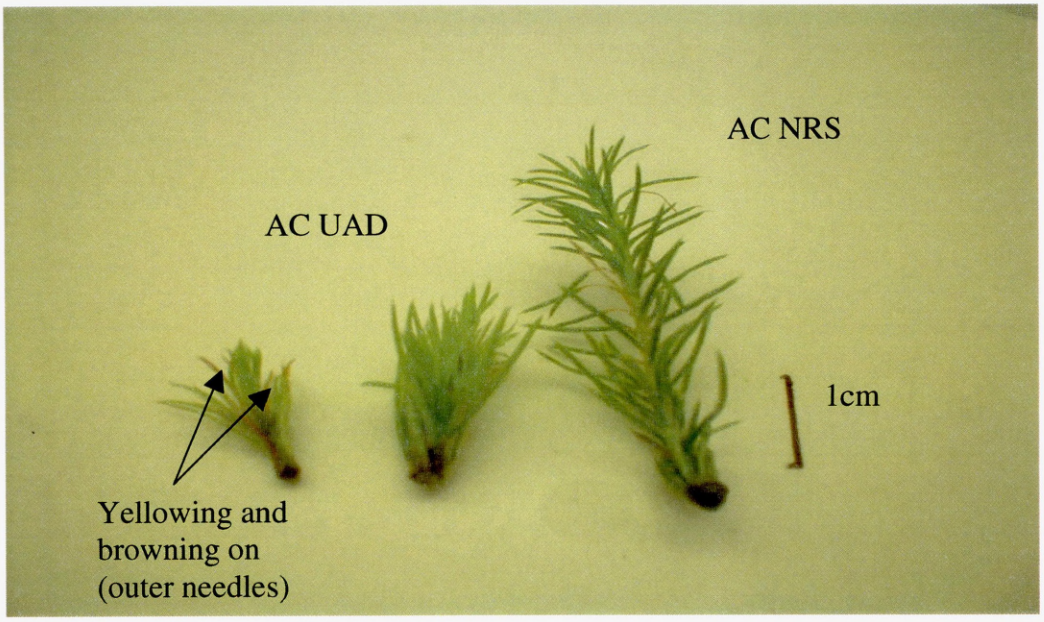

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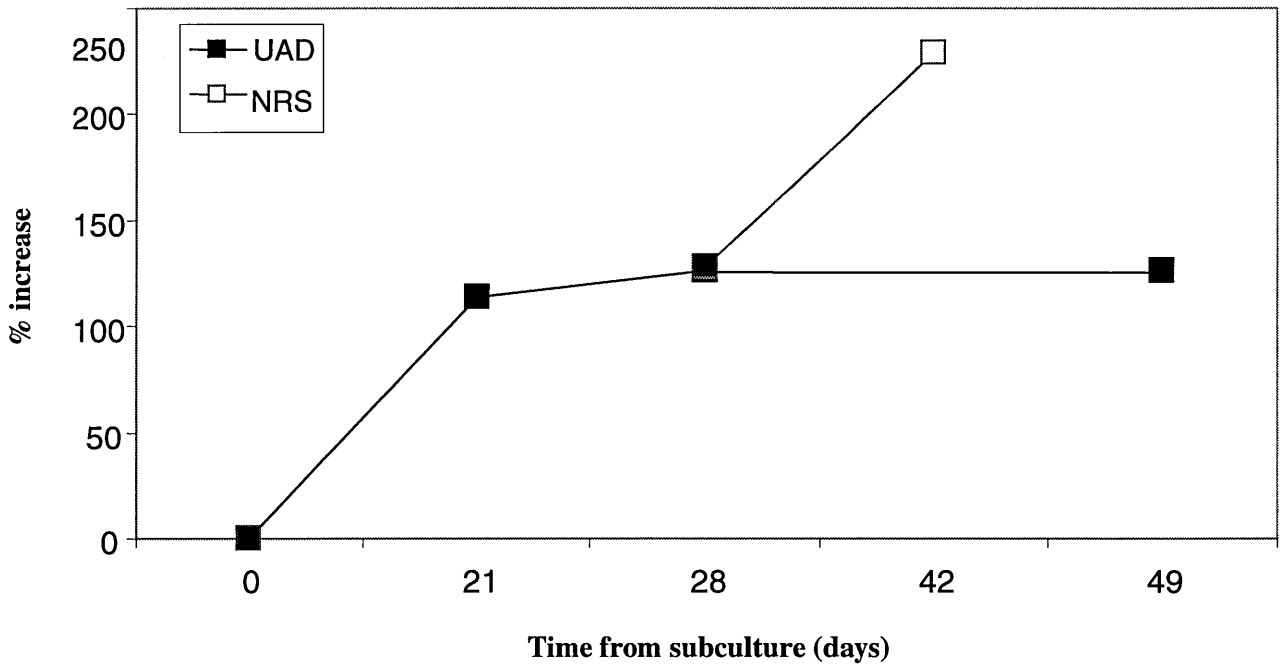


Figure 4.6 A comparison of accumulative extension (cm) in UAD and NRS shoot-tips

Growth as a percentage of original subculture length in genotype AC during culture in growth rooms located at UAD (University of Abertay Dundee) and NRS (Northern Research Station, Forestry Commission). N= 10 ± SEM

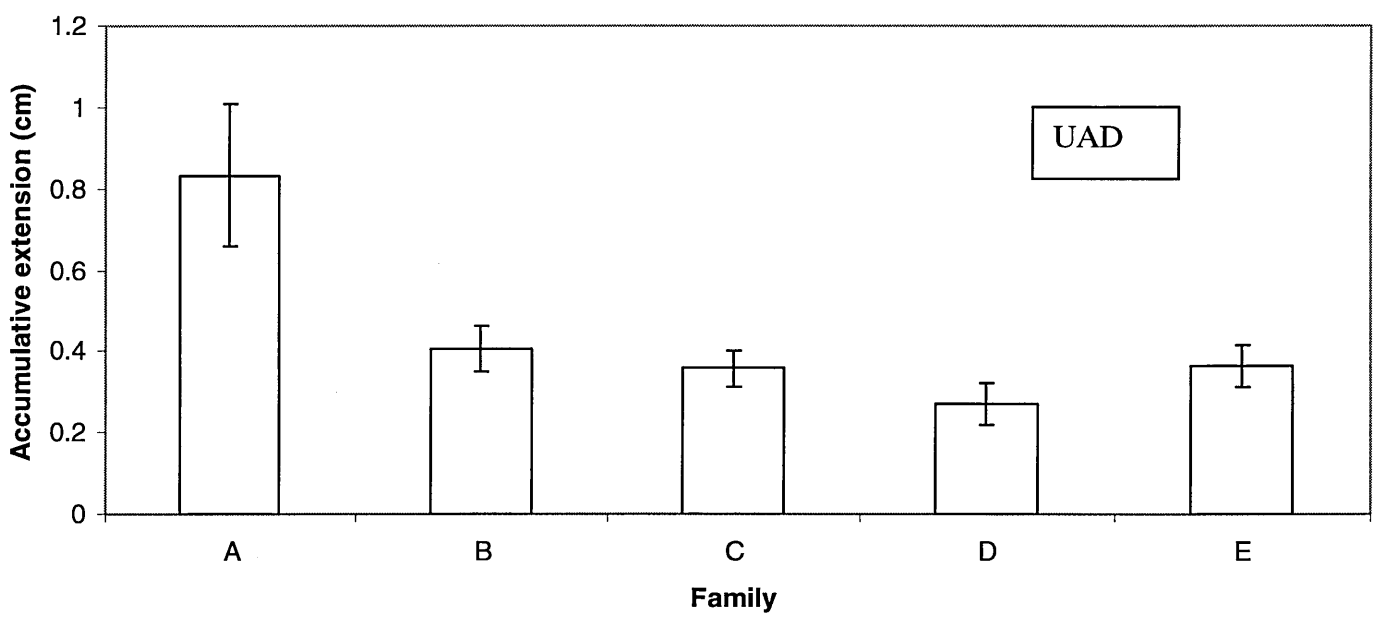
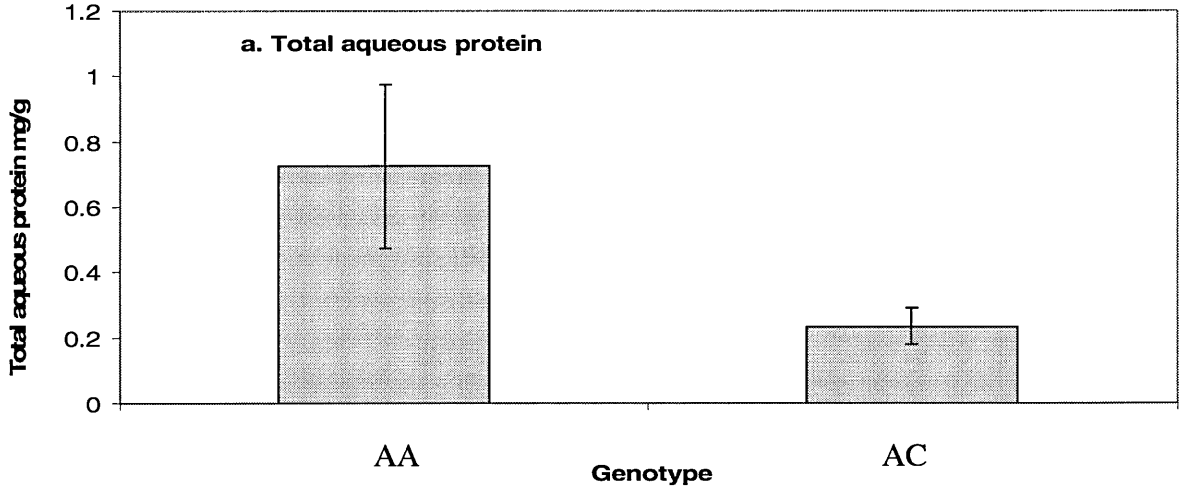
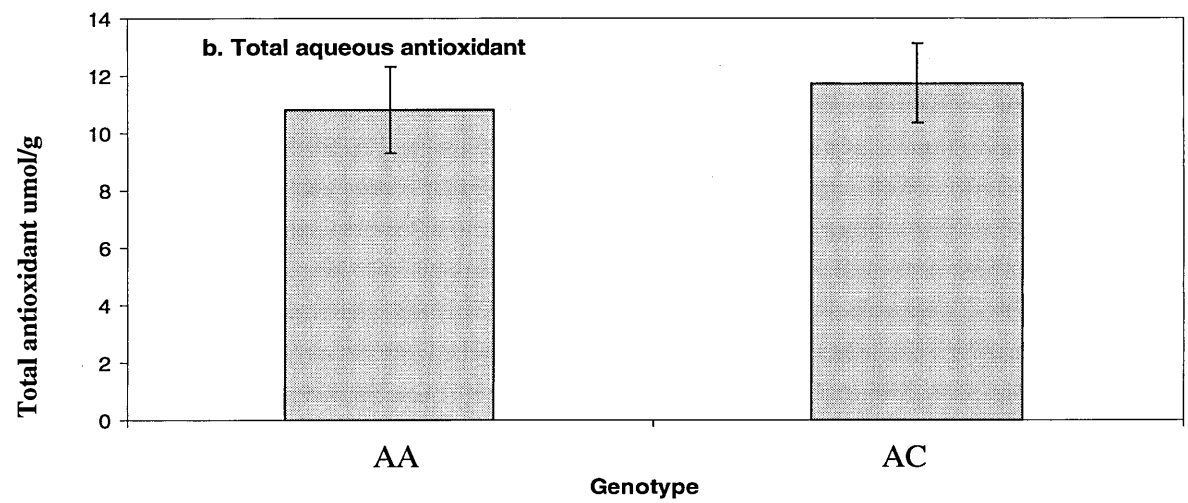


Figure 4.7 A comparison of accumulative extension (cm) between genotypes

families A-E. N=20-50 ± SEM, after 28 days in UAD culture.

4.3.2 Biochemical markers





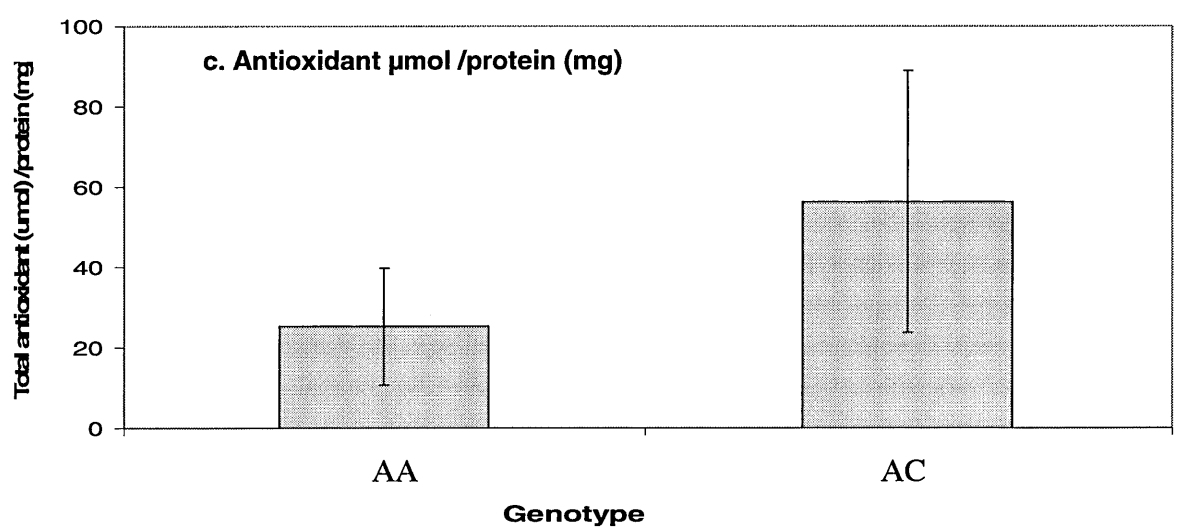


Figure 4.8 Genotype, antioxidant and protein comparisons
(a) Total water-soluble protein content (mg/g), (b) Total aqueous antioxidant capacity (µmol/g) and (c) total antioxidant/total protein (µmol/mg) from shoot-tip cultures of genotype AA and AC from UAD. Values are means $\pm$ SEM of 3 replicate extractions per sample. No statistical differences between pairs using Students paired t-test $P<0.05$.

Protein and antioxidant determinations indicated that there were no significant differences ($P>0.05$) between shoot-tips of genotype AA and AC cultured at UAD (Fig. 4.8). Shoot-tip cultures from genotype AA showed ca. 0.4mg/g more protein than genotype AC. On a total antioxidant/ protein basis (Fig. 4.8c) genotype AC shows double the µmol/mg than genotype AA.

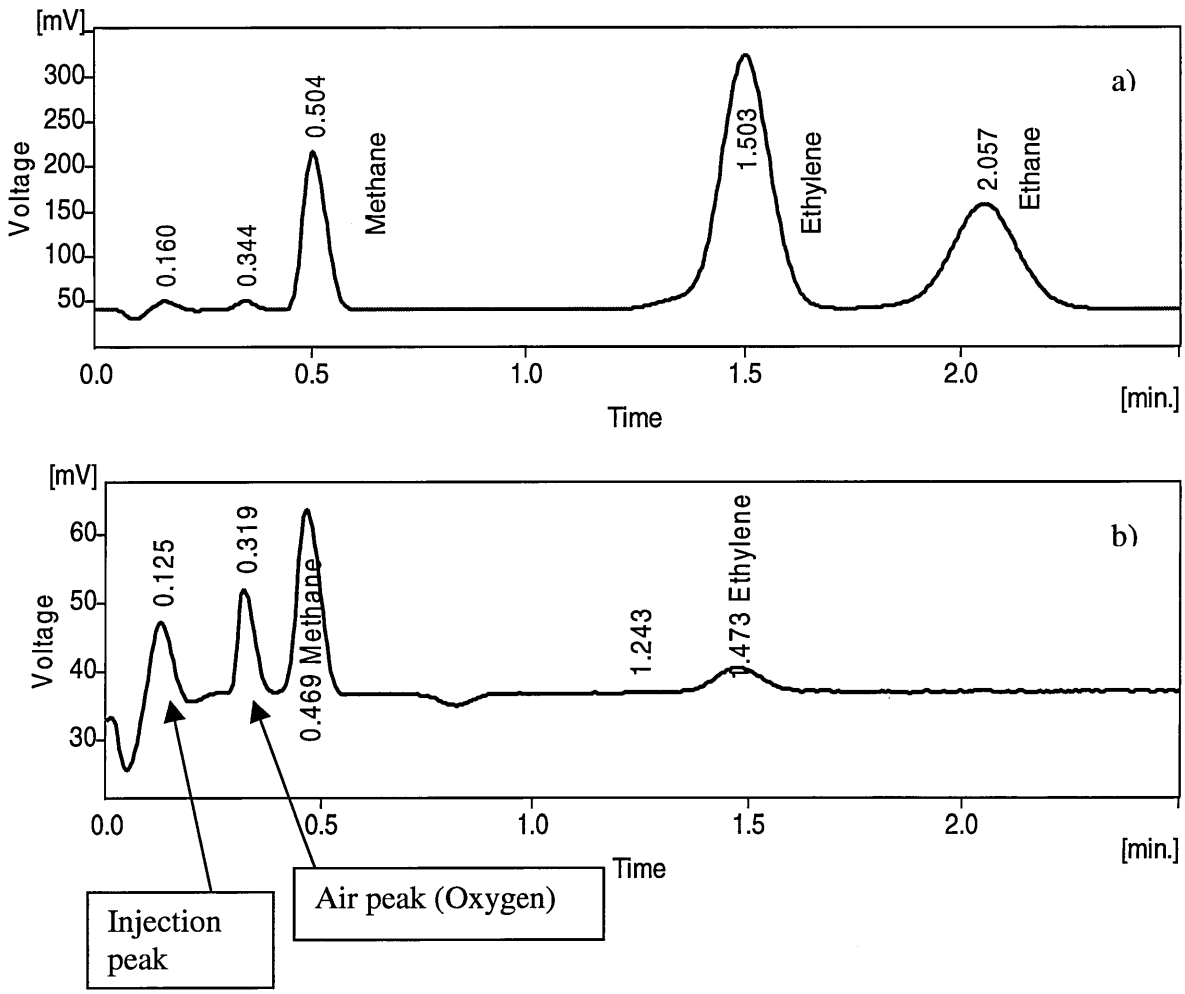


Figure 4.9 Gas chromatograms showing methane, ethylene and ethane (standard only) peaks (a) Standard Scotty I analysed gases, product number 2-2566. Standards consisted of ethylene 30ppm, ethane 15ppm, methane 15ppm. (b) UAD AC 3= University of Abertay, Dundee, genotype AC, replicate 3

Volatile hydrocarbons evolved from the headspace of shoot-tip cultures (sealed for ca.48hr) of genotype AC, previously cultured for 6 months at UAD or NRS were compared. Figure 4.9, shows that ethylene was the only volatile hydrocarbon to accumulate above background levels following sealing. No significant differences (P>0.05) in ethylene evolution between genotypes or locations were determined (Fig. 4.10).

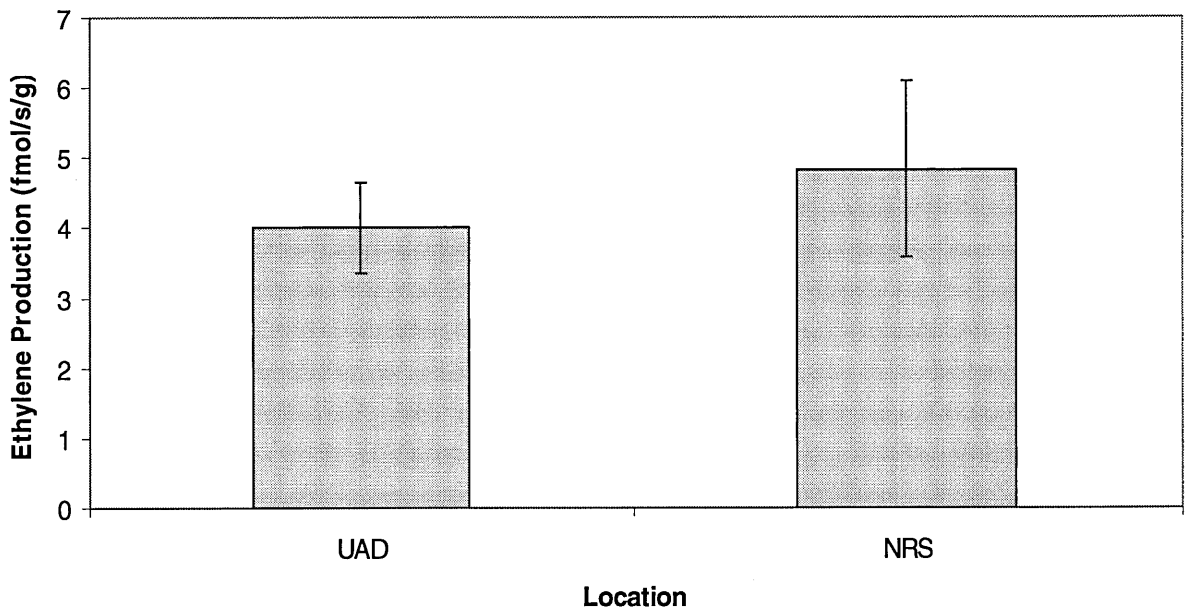


Figure 4.10 Comparisons in ethylene production (fmol/s/g) in shoot-tip cultures.
Genotype AC, from UAD (University of Abertay, Dundee) and NRS (Northern Research Station, Roslin). Values are means ± SEM of 3 replicate extractions per sample. There were no significant differences (P>0.05) between groups using a 1– way ANOVA in Minitab vs. 14.

4.3.2.1 DNA methylation

The DNA methylation status of the shoot-tip extractions from genotype AA previously cultured at UAD and NRS was investigated. Nucleosides were detected through HPLC analysis and compared with nucleoside standards for DNA, RNA and 5-methyl-2'deoxycytidine (Fig.4.12). There were no significant differences (P>0.05) between UAD and NRS in any of the methylation results (Table 4.5). There was no significant correlation (P>0.05) between % deoxycytidine methylation and % RNA content (Fig.4.11).

Table 4.5 DNA Methylation results

	% methylation in deoxycytidine	% methylation in DNA	% GC in DNA	% nucleic acid = RNA
UAD	15.7±0.4	3.1±0.1	42.1±0.6	95.3+0.7
NRS	16.2±0.4	3.2±0.1	41.6±0.5	92.9+1.1

% GC in DNA= % guanosine and cytidine in DNA

UAD =University of Abertay Dundee; NRS=Northern Research Station, Roslin

Values are means ± SEM from 3 replicate extractions. There were no significant difference (P>0.05) between locations.

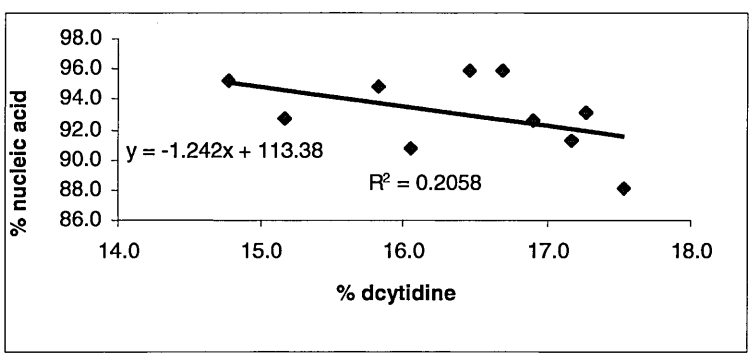


Figure 4.11 % Deoxycytidine methylation and % RNA content

No significant correlation (P<0.05) using Pearsons Correlation test Excel vs.9 was observed

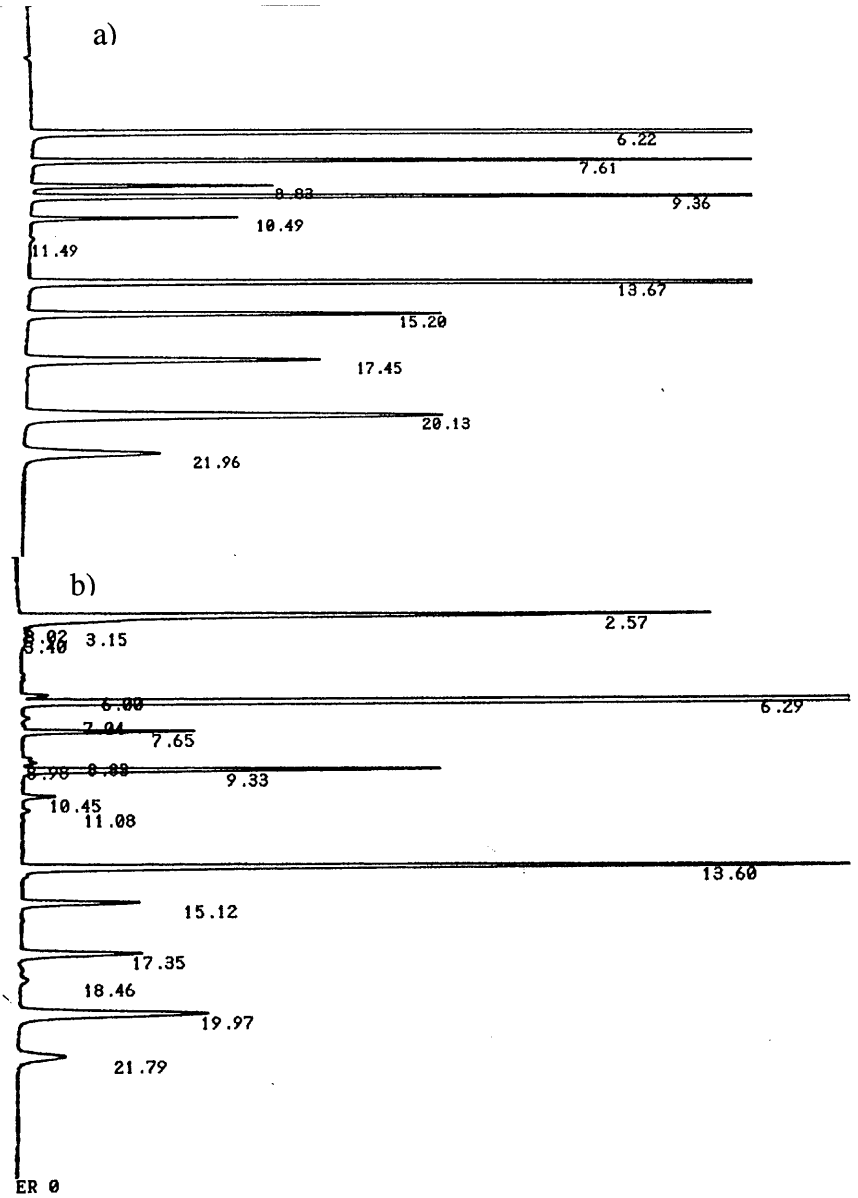


Figure 4.12 Examples of HPLC chromatograms
a) Standard 3 = 150 µM RNA nucleoside, 50 µM DNA nucleoside, 15 µM 5-methyldeoycytidine/5-methylcytidine b) UAD AC 3= Shoot culture genotype AC, replicate 3
Retention times and corresponding nucleosides:
6.2 = CYTIDINE; 7.6= DEOXCYTIDINE; 8.0 =METHYLCYTIDINE;
9.3= URIDINE; 10.4= METHYLDEOXYCYTIDINE; 13.6= GUANOSINE;
15.1-15.2=DEOXYGUANOSINE; 17.3-17.4= THYMIDINE;
19.9-20.1= ADENOSINE; 21.7-21.9= DEOXYADENOSINE

4.3.3 Pre-culture optimisation

Cold hardening was optimised for an encapsulation-dehydration protocol (Fig. 4.13). One week and two weeks pre-cultured shoot-tips showed control comparable survival and re-growth. Shoot-tips that were pre-cultured in the cold for longer (4-6wks) survived but did not grow. They also exhibited physiological differences such as yellowing and 'bunching' of needles around the shoot-tip base.

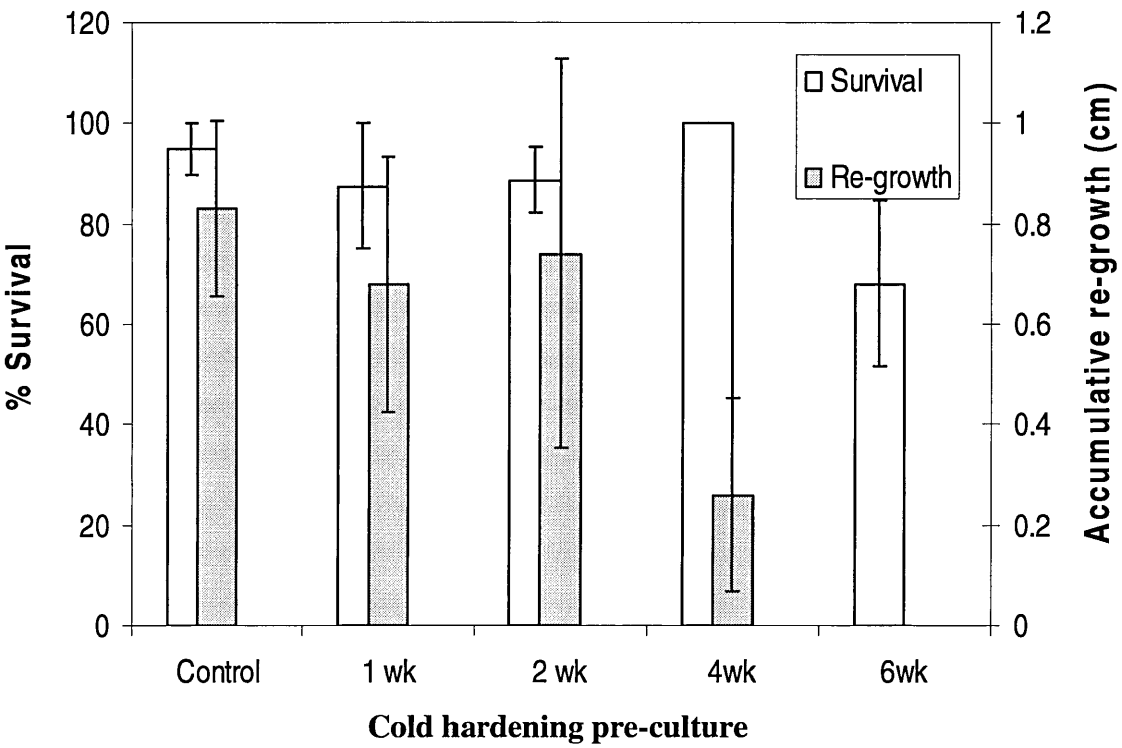
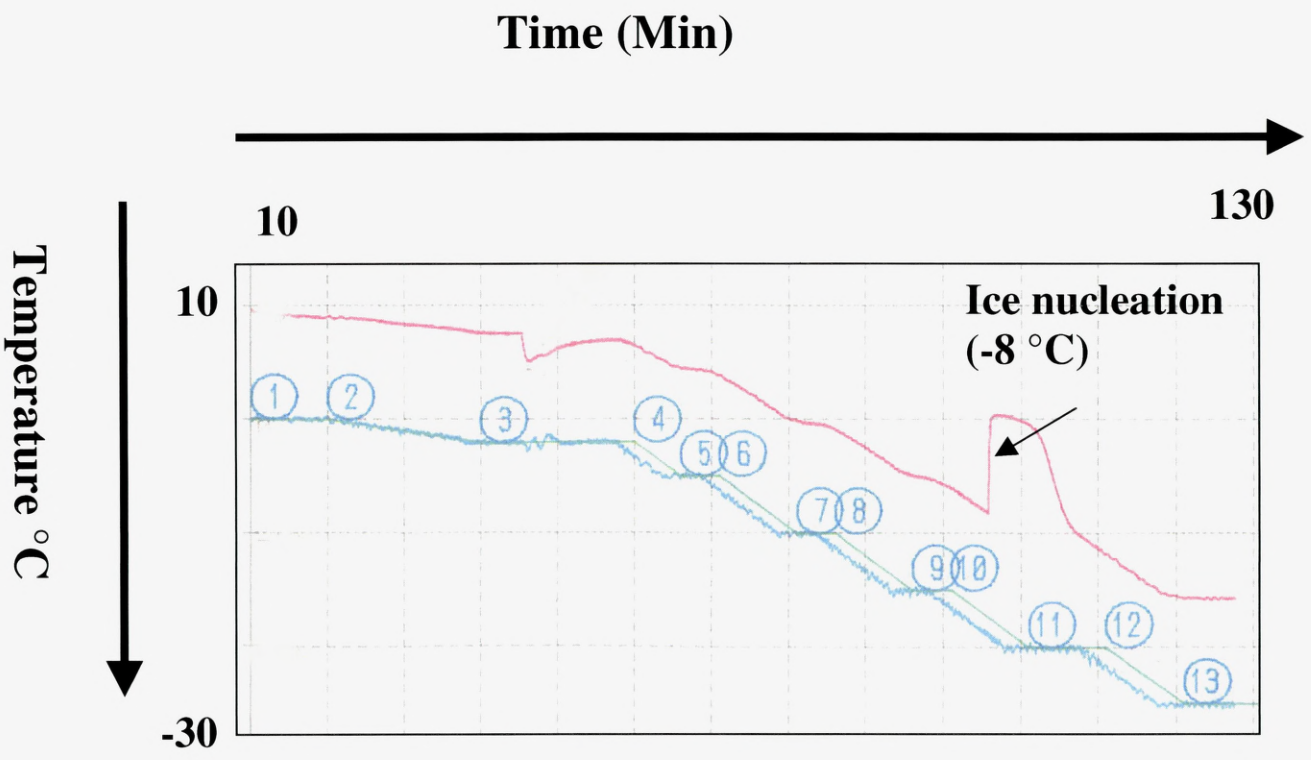


Figure 4.13 The effect of cold pre-cultures on apical shoot-tip apices % survival and re-growth (cm) 28 days after pre-culture.

Control = no cold pre-culture. 1 wk= cultures placed in dark at 4°C; 2-6wks cultures placed in dark at 4°C for 8hr and in 20 ±2 °C and light for 16hr. % survival data points represent 5 replicate shoot-tips per experiment, experiment repeated 4 times to produce mean ± SEM. Re-growth data points represent mean ± SEM of 5 replicate shoot-tips.

4.3.3.1 Controlled rate cooling of cold hardened (sucrose) shoot-tips

In vitro shoot-tip apices were cold hardened for 1 week at 4ºC in either MS medium or MS medium supplemented with 5-11% (w/v) sucrose and their cold hardiness was tested during slow cooling to -25ºC. The program printout, using a probe inserted into a cryovial with tissue paper damped with liquid MS (the control cryovial environment) showed ice nucleation occurring at -8ºC (Fig.4.14).



Pink line= Temperature inside cryovial (from probe)
Blue line= Ambient temperature inside Programmable freezer
Green line= Programmed temperature profile
Numbers represent program steps

Figure 4.14 Programmable freezer (KRYO 10 PLANAR) printout.

Pink line represents profile from probe placed in control cryovial (MS moistened tissue paper). Green and blue lines show programmed hold where cryovials were removed to sample the effect of temperature on pre-cultured shoot-tips.

The mean % survival and accumulative re-growth (cm) of shoot-tip apices following 1 wk cold pre-culture with 0-11% (w/v) sucrose and following cooling to 0, -5 and -10ºC is shown in Table 4.6. At 0 to –5ºC cooling 100% survival was observed in all replicate shoot-tips,

although re-growth was variable from 0 to 0.6cm over 28 days. No survival was observed following cooling (48hr) to -15°C. Only shoot-tips that had been pre-treated with 7-11% (w/v) sucrose survived cooling to -10°C, although no subsequent re-growth was observed from these shoot-tips.

Table 4.6 Controlled rate cooling response

		% Sucrose (w/v)				
	T°C shoot-tips removed	**0**	**5**	**7**	**9**	**11**
% survival 48hr	0	100± 0	100±0	100±0	100±0	100±0
	-5	100±0	100±0	100±0	100±0	100±0
	-10	0	0	35±25	30±10	50±10
Accumulative growth (cm) 28 days	0	0.6±0.1	0	0	0.6±0.5	0
	-5	0.4±0.5	0.6±0.6	0.2±0.1	0.4±0.1	0
	-10	-	-	0	0	0

% survival = Mean % survival after 48hr following pre-culture treatment ± SEM derived from 5 replicate shoot-tips per experiment, 2 experiments.
Accumulative growth= Mean accumulative extension (cm) from 5 replicate shoot-tips. - = Not available no shoot-tips alive.

4.3.3.2 Pre-culture treatment optimisation

The greatest mean post-LN survival (20%) was observed in shoot-tips pre-cultured in 9% sucrose for 1 week, (Fig. 4.15), 28 days after treatment. These shoot-tips were the only post-LN shoot-tips to re-grow. One shoot-tip apex survived for a further 20 days before the onset of necrosis (Fig. 4.20 c-f). Control cultures showed 100% re-growth without LN immersion and 0% re-growth following LN. Sucrose cultured shoot-tips showed 50-90% recovery without LN, but only the 9% (w/v) sucrose treated shoot-tip survived LN immersion. Trehalose treated shoot-tips showed no post LN recovery and 11% (w/v) trehalose treated shoot-tips did not recover following any treatment. Post LN recovery (10%) was observed following 5% (w/v) proline and 10^{-4}M ABA, but these cultures remained dormant and did not re-grow. Without LN immersion, the post-ABA treated shoot-tips showed 40-50% recovery.

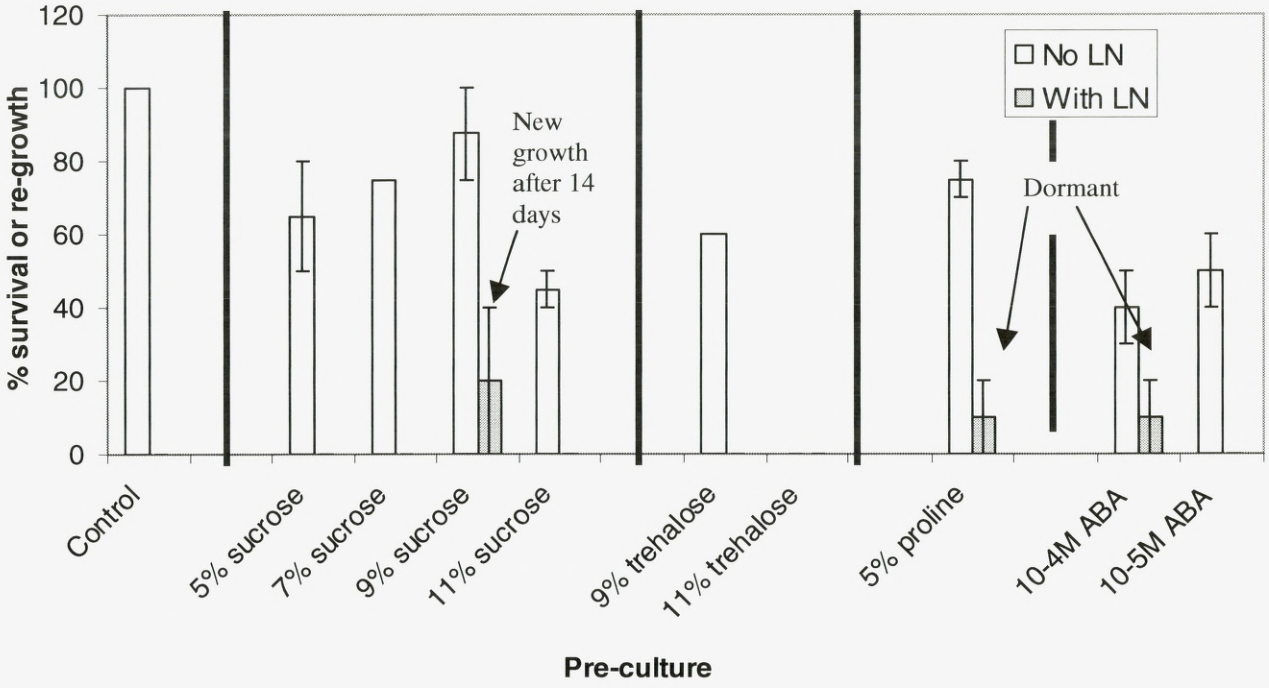


Figure 4.15 Shoot-tip apices cryopreservation with pre-culture additives

Following 1 week cold hardening and pre-culture additives (sucrose, trehalose, proline and ABA), 4hr laminar air-flow desiccation, with and without LN immersion. Recovery after 28 days n=5 replicate shoot-tips per experiment, duplicated for mean $\pm$ SEM.

4.3.4 DMSO pre-treatment

Dissected shoot-tip apices were treated with DMSO (1-5% v/v) for 24 hr and the growth response monitored. After 28 days, shoot-tips treated with 5% (v/v) DMSO showed 100% recovery and survival; a 10% improval to the mean of the control culture response, (Fig. 4.16). Figure 4.17 shows that the 5% (v/v) DMSO treated shoot-tips showed visibly improved growth compared to the control cultures, but retained comparable morphology. Shoot-tips were then cultured in 9% (w/v) sucrose for 1-week in continuous (4 °C) cold treatment), followed by 5% (v/v) DMSO post-dissection treatment, encapsulation and 4hr laminar air-flow desiccation and LN immersion and showed 20% recovery from 5 replicate shoot-tips tested (data not shown because identical to Fig. 4.16; no SEM margin because of limited shoot tip availability, experiment undertaken only once).

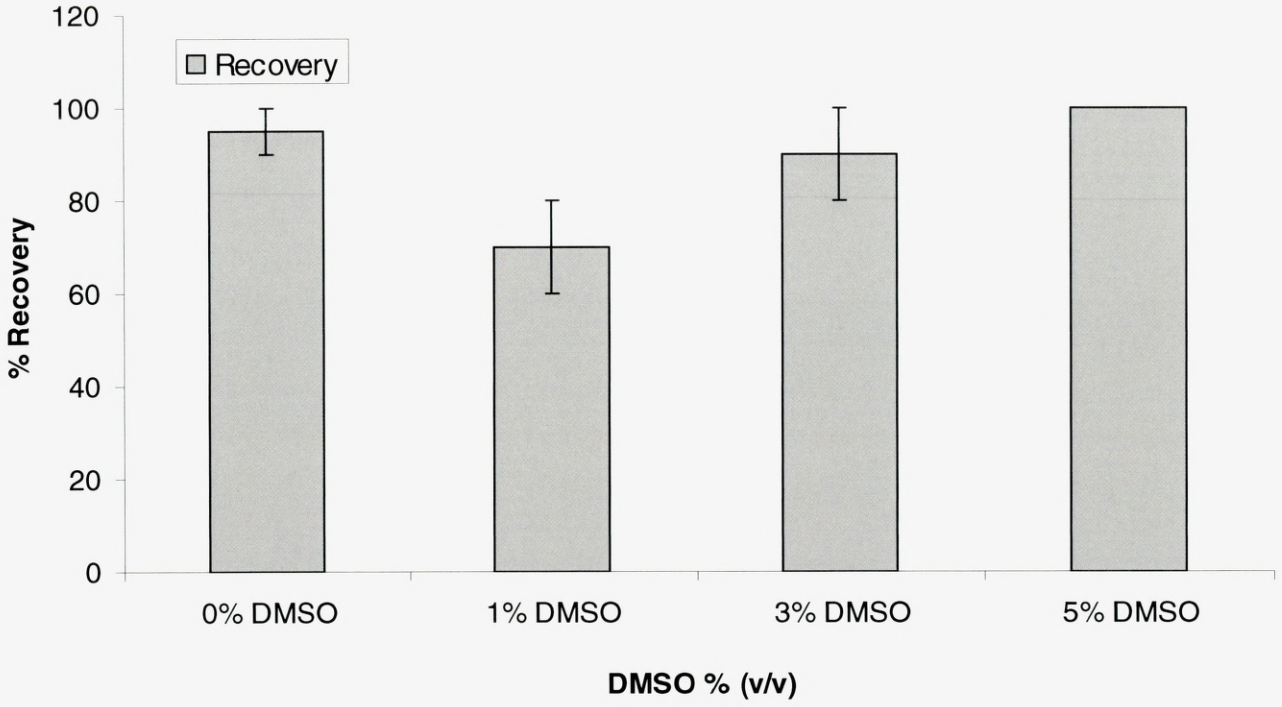


Figure 4.16 The % recovery of dissected shoot-tip apices following DMSO treatments DMSO treatment, (0-5% 24hr) 28 days after treatment. N= 5 replicate shoot-tips apices per experiment, duplicated= mean ± SEM.

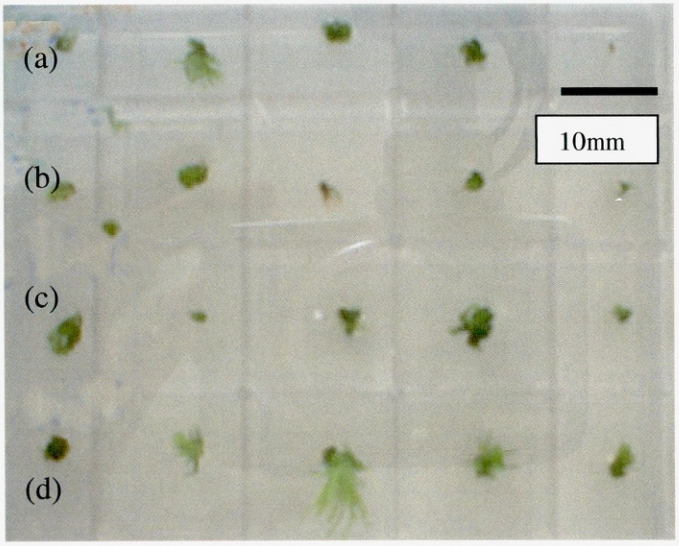


Figure 4.17 Image showing effects of DMSO treatment on dissected shoot-tip apices. 24hr exposure (a) 0% (v/v) DMSO, (b) 1% (v/v) DMSO (c) 3% (v/v) DMSO (d) 5 % (v/v) DMSO

4.3.5 Shoot-tip meristem excision and encapsulation-dehydration

Active apical shoot-tip apices and dormant lateral buds were dissected to three sizes (1-4mm in length) and sampled following treatment from several stages of encapsulation-dehydration. Dissected size 1 and 2 apices showed a mean of 90-100% green area, but this was reduced in size 3 spices to 70% (Fig. 4.18). Encapsulated shoot-tip apice, showed reduced green area (40% less) in all three sizes, by day 49, but did show survival and the bead did not appear to prevent growth (Fig.4.20a). All sizes of shoot-tips that had undergone encapsulation with sucrose and air-desiccation and shoot-tips that were immersed in LN were dead after 49 days, (Fig. 4.18).

Following 49 days, lateral size 1 buds without LN treatment showed a mean green area of 60 % (Fig. 4.19). This was reduced to 30% in size 2 buds and no survival was observed in the size 3 buds. At least 10% of lateral buds were alive following LN immersion at 49 days, but did not subsequently re-grow.

4.3.6 Dehydration and desiccation parameters

The laminar air-flow desiccation time was optimised through % relative moisture determination and post-desiccation survival. When beads were osmotically dehydrated (with sucrose) before laminar air-desiccation, a RM content of 25 to 30% was produced (Fig. 4.22a). When sucrose dehydration was excluded the RM content was 10% higher and the rate of water loss faster between 3 and 5 hr. Encapsulated, sucrose dehydrated, shoot-tips did not survive following >1hr of laminar-air-flow desiccation, but encapsulated shoot-tips pre-cultured for 1 week at (4°C) showed 60% survival after 4hr of desiccation and 30% following 6 hr, (Fig. 4.22b).

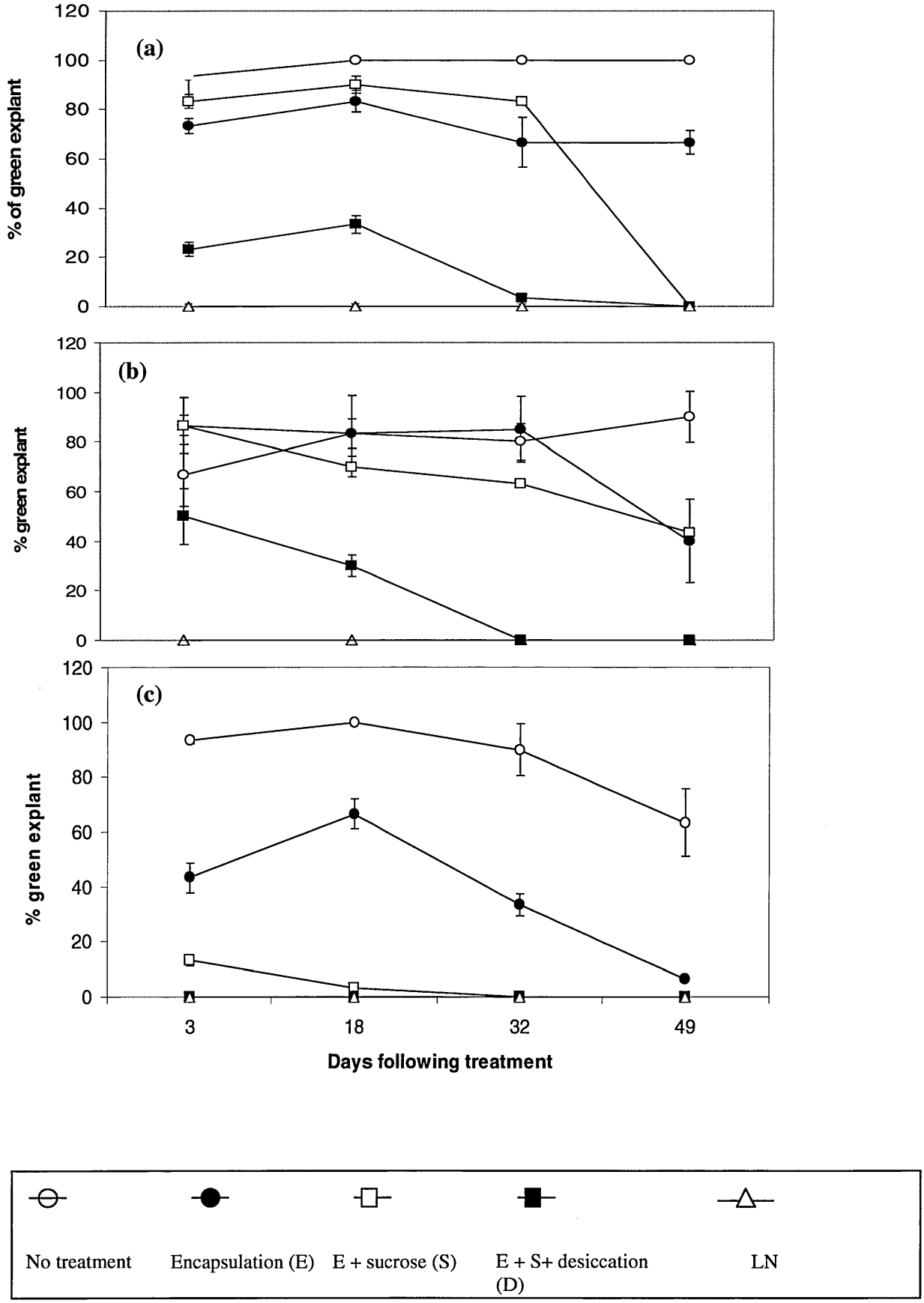


Figure 4.18 Mean % of green apical shoot-tips following cryopreservation treatment size 1-3.
(a) size 1 shoot-tip apex dissection, (b) size 2 shoot-tip apex dissection, (c) size 3 shoot-tip apex dissection. N= 3 replicate shoot-tip apices ± SEM. E=Na-alginate encapsulation, E+S= Encapsulation + sucrose, E+D= Encapsulation + sucrose + laminar air-flow desiccation, LN = All pre-treatments + LN immersion for 24hr. Data sampled over 49 days.

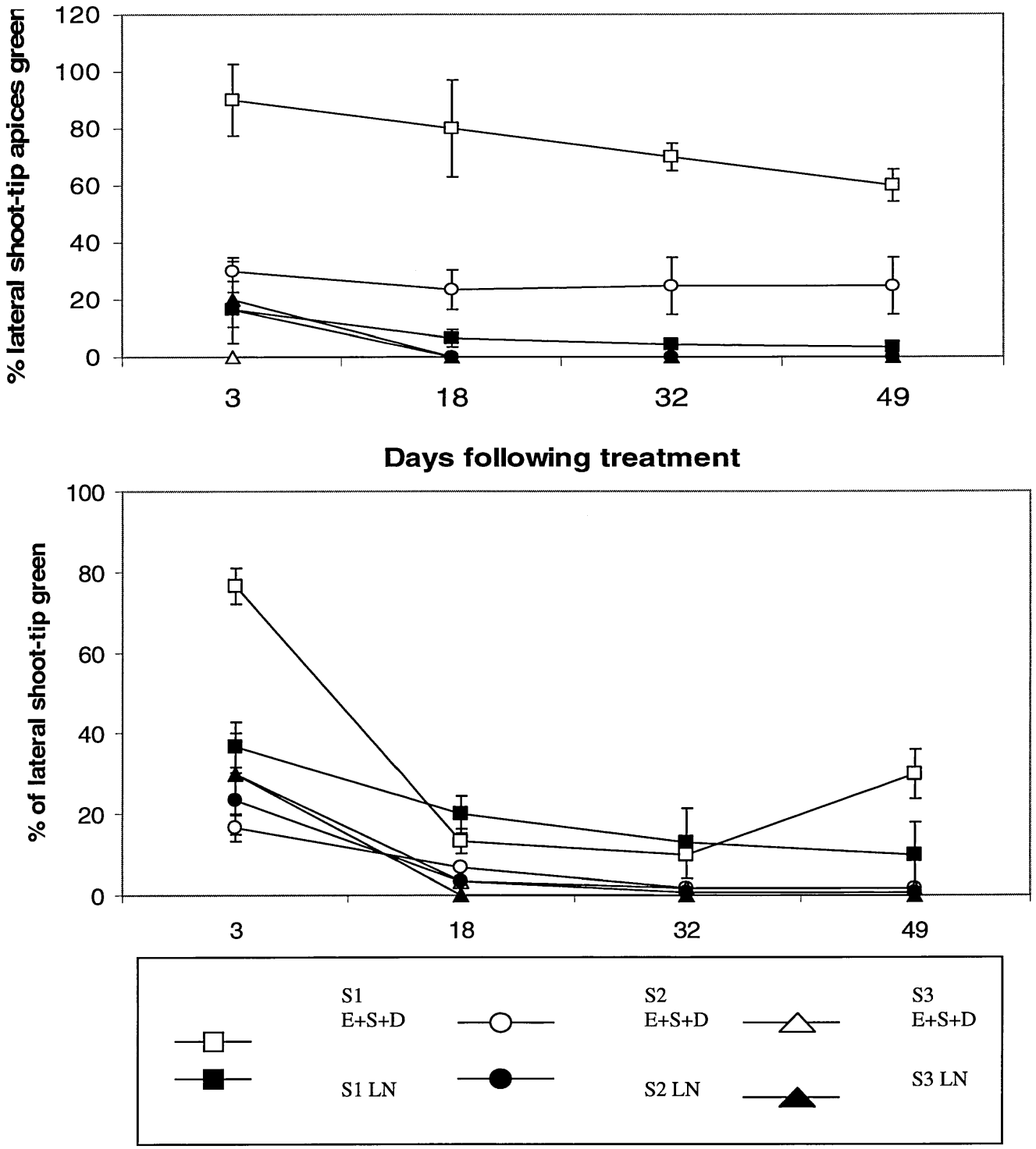


Figure 4.19 Mean % green lateral shoot-tips following cryopreservation treatment
49 days of culture (a) shoot-tips kept in the bead, (b) shoot-tips dissected out of bead on day 3. N=3 replicate shoot-tips ± SEM : E=encapsulation, S=sucrose, D=desiccation LN=liquid nitrogen

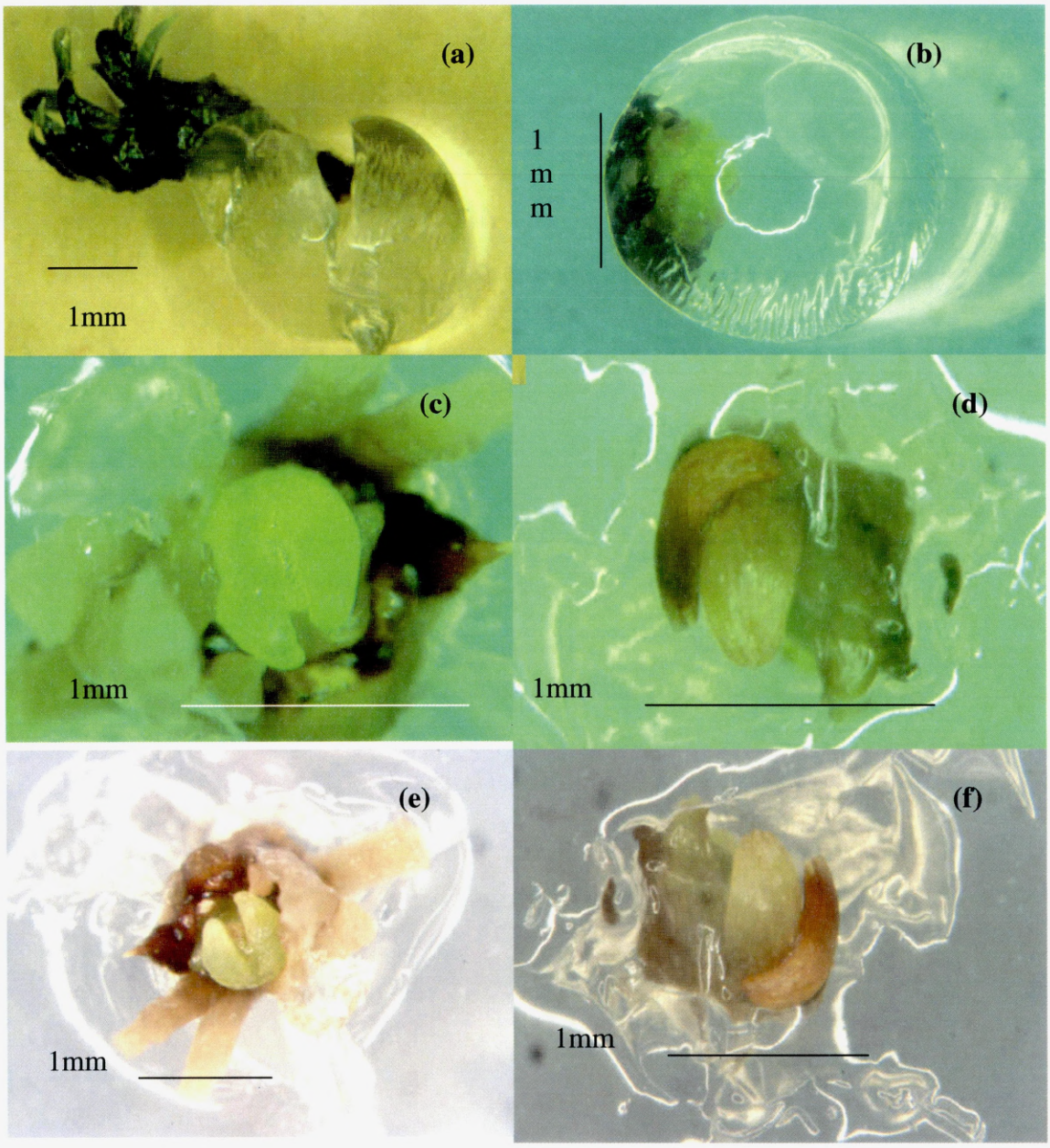


Figure 4.20 Images of shoot-tip apices

Following (a) shoot growing free of Na-alginate matrix, 28 days after encapsulation, (b) green meristematic mass from encapsulation-desiccation treatment (28 days after treatment) mass did not differentiate or proliferate further and was necrotic after 42 days.(c) Post-LN meristem treated with 9% sucrose +1 week cold-pre-culture 1 week, (dissected out of bead at day 28) meristematic dome and primordia green became protected by bud scales, (d) - (f) further degradation over following 20 days until all shoot material necrotic and died.

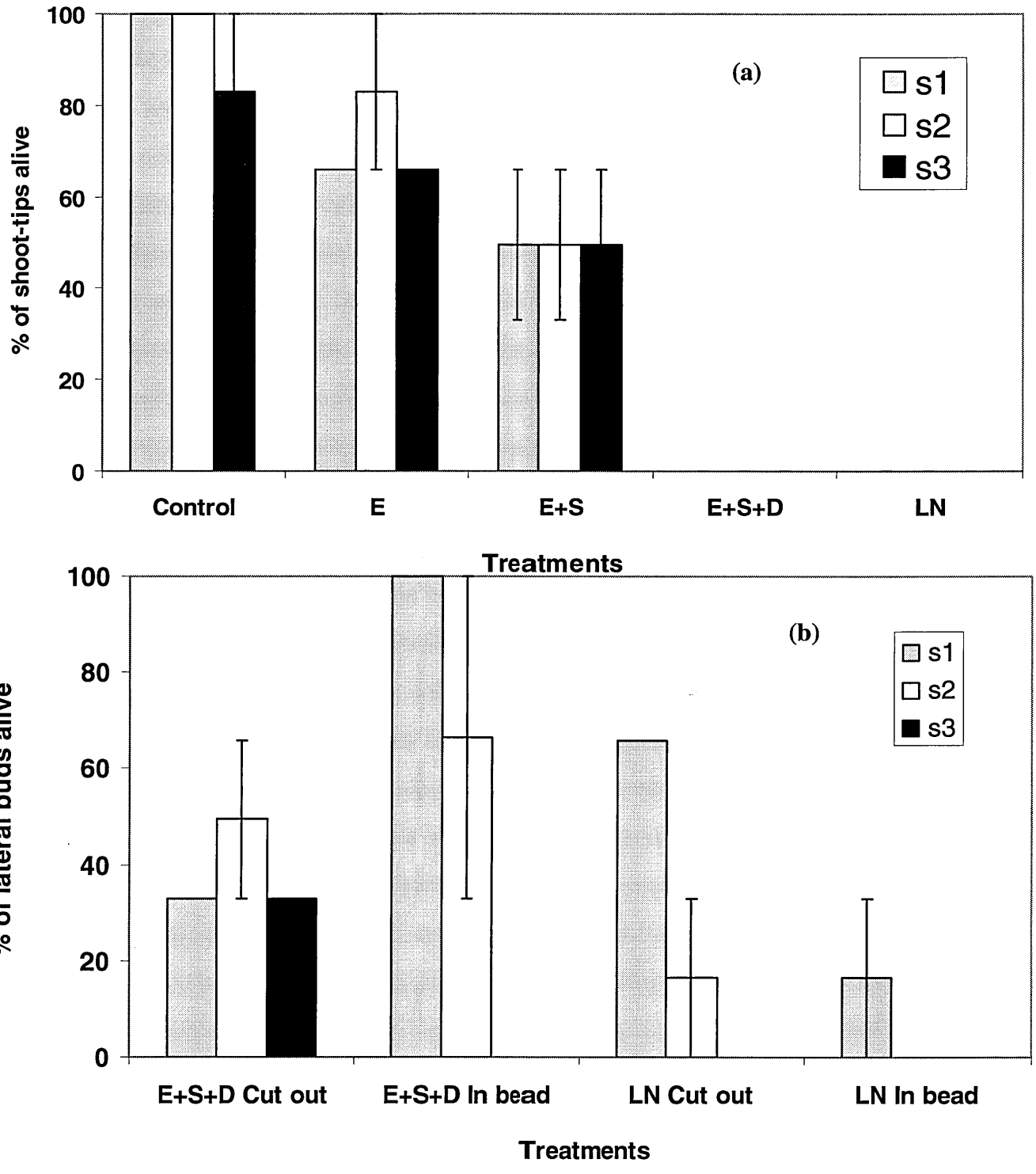


Figure 4.21 Mean % shoot-tip meristems showing post cryopreservation treatment recovery

Total % of (a) apical and (b) lateral buds (left in or cut out of bead at day 3) after 48 days of recovery
Where no SEM bars present replicate experiments show same mean value.
S1=size 1, S2=size 2, S3 =size 3 meristems
E=Na alginate encapsulation, S= 0.75M sucrose 18hr dehydration, D=laminar air-flow desiccation

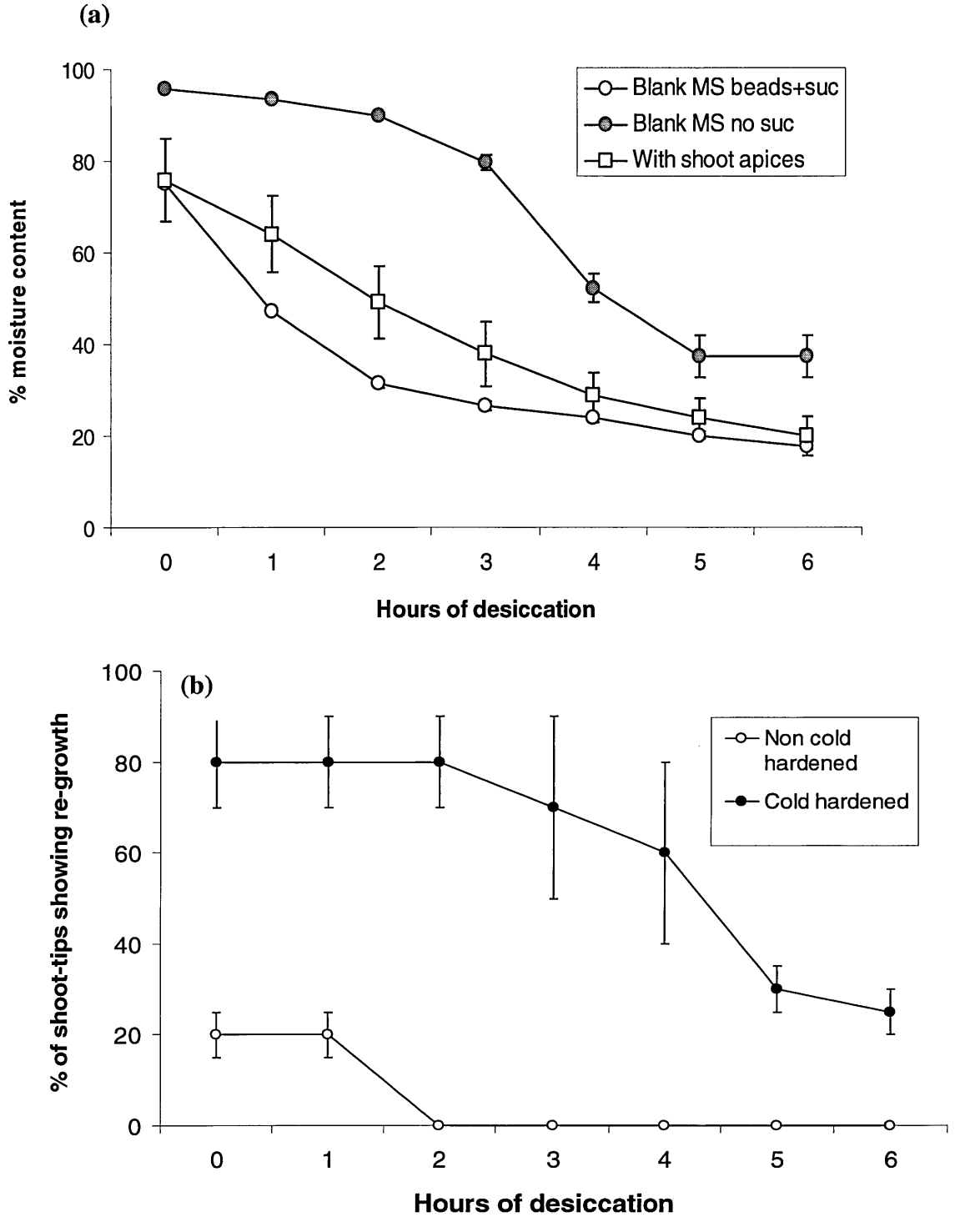


Figure 4.22 Relative moisture content and survival of encapsulated shoot-tip apices following 6hr of laminar air-flow desiccation

(a) % Relative Moisture content of beads following laminar air-flow desiccation, n=10 replicate beads ± SEM. (b) % Na-alginate encapsulated shoot-tip apices showing re-growth following laminar air-flow desiccation following no pre-culture and following 1 week of cold pre-culture (4 °C) recorded after 28 days of post-cryopreservation culture. n= 10 replicate encapsulated shoot-tip apices per experiment, experiment duplicated

4.3.7 Encapsulation-vitrification

Following osmoprotection alone shoot-tips showed 50% survival at day 7, recovering to 60% by day 28 (Fig. 4.23). Shoot-tips treated with PVS2 for 1-2hr had reduced survival. Shoot-tips treated with PVS2 for 30min showed increased survival (80%) on day 7 but this was reduced to 50% by day 28. No shoot-tips survived LN immersion regardless of the pre-treatment.

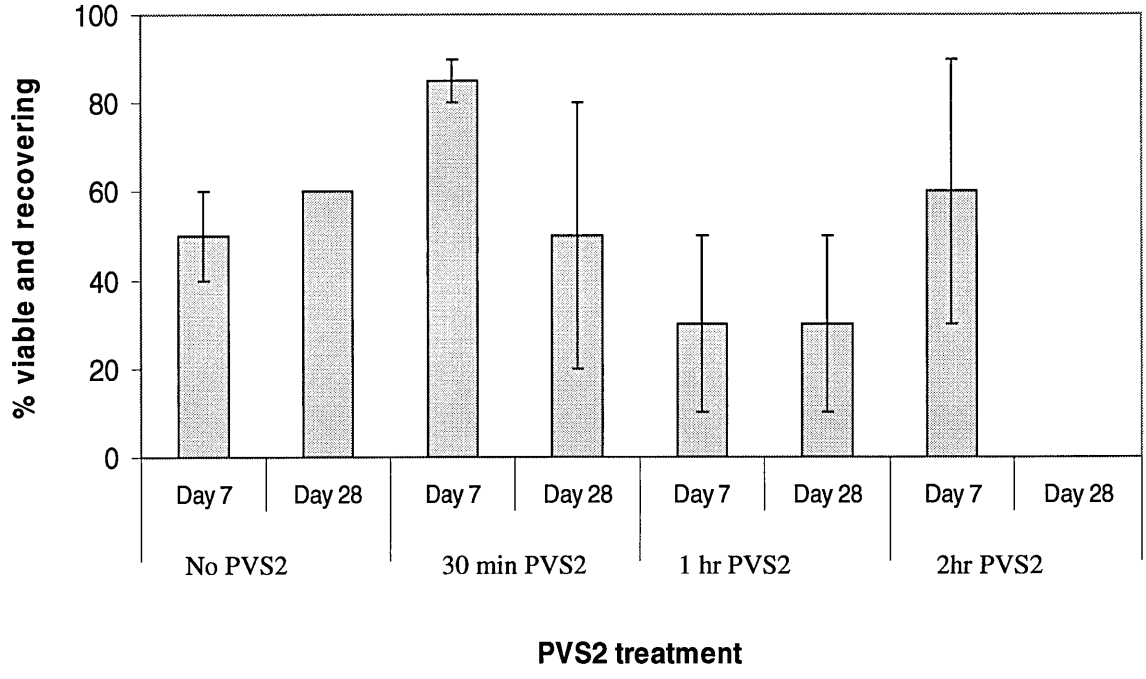


Figure 4.23 Number of encapsulated shoot-tip apices alive at 7 days and showing recovery at 28 days following PVS2 treatments.

Mean ± SEM derived from 5 replicate shoot-tips per experiment, experiment undertaken twice. No shoot-tips alive or recovering following PVS2 treatment + LN immersion.

4.3.8 Post-cryopreservation optimisation

Dissected shoot-tip apices were cultured on MS medium supplemented with different plant growth regulators (PGRs) for 7 days under routine culture conditions, prior to transfer back to standard MS medium. The number of shoot-tips alive and recovering was monitored at 14 and 28 days following PGR culture (Fig. 4.24). Dissected shoot-tips without PGR treatment showed 100% recovery, but all shoot-tips treated with PGRs, or with the solution used to dissolve the PGRs in prior to filter sterilisation and addition to medium, showed inhibited growth. Shoot-tip apices cultured in 1M GA_3 showed <10% recovery following 28 days. TDZ (0.5M) cultured shoot-tips showed the best recovery (80% still viable by day 28). Figure 4.21 shows survival was improved when buds were maintained in Na-alginate beads following treatment.

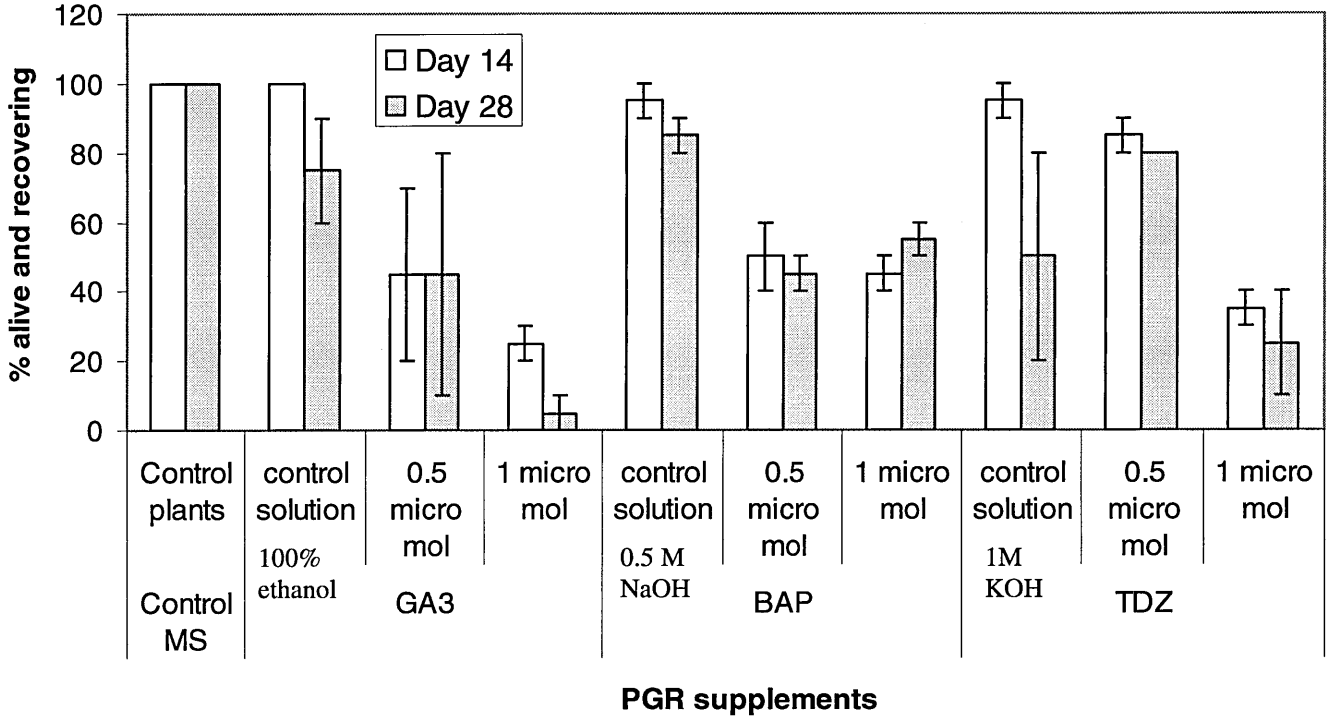


Figure 4.24 The effect of plant growth regulator supplements (GA_3, gibberelic acid, BAP, 6-benzylaminopurine, TDZ, thidiazuron) on dissected shoot-tip apices survival at 7 days post-treatment and recovery at 28 days. Mean ± SEM determined from duplicated experiments of 5 replicate shoot-tip apices per treatment.

4.4 Discussion

The development of conifer *in vitro* shoot-tip cryopreservation has been progressed and for the first time it is reported that shoot-tips survived cryopreservation. There are indications towards the steps required to assist in full post-cryopreservation recovery and plantlet regeneration. These steps also further understanding of *in vitro* recalcitrance, applying biochemical markers to assess free radical damage and DNA methylation and suggest recommendations for genebanking and ameliorating deleterious phenolic accumulation. Cryogenic protocols, incorporating cold hardening, pre-culture additives, wounding injury amelioration and post-cryopreservation recovery culture were developed to improve shoot-tip post-LN survival. All methodology is suitable for implementation at the partner institute NRS should optimisation be continued.

4.4.1. *In vitro* standardisation and recalcitrance

Physiological indicators for inter-locational and genotypic differences in growth were observed. Shoot-tips that had been initiated and maintained at the UK Forestry Commission's Northern Research Station (NRS) facility were transferred to equivalent parameter growth room facilities at the University of Abertay Dundee (UAD). After 6 months of culture the growth rate of shoots from the same genotype (AC) after 42 days at NRS was observed to be nearly double that of shoots at UAD (Fig. 4.6). UAD cultures also exhibited other physiological differences including extended internodes, yellowing and bunching of needles (Fig. 4.5).

UAD and NRS culture rooms shared fixed lighting, temperature and relative humidity parameters, yet growth differences in identical cultures subcultured to the same size on the same day existed. Probes inserted in the UAD culture tube medium indicated an increased temperature of + 2 to 4°C. It is believed that this was in part due to the use of a different shelf material. There are reports of *in vitro* culture differences influencing hyperhydricity and oxidative stress. Relative humidity, hyperhydricity, dry weight, multiplication rates and the activity of the antioxidant enzymatic system changed in carnation shoot cultures, in relation to the agar concentration used and in relation to the use of a tube cooling system (Saher, *et al,* 2005). Hyperhydricity and lipid peroxidation decreased when a cooling system was applied.

Another possible micro-environmental recalcitrance induction pathway may be associated with the proximity and intensity of the UAD UV lighting tubes as compared to the NRS growth room. It has been reported that young emerging conifer needles may not be as well protected from UV-B radiation, as mature needles that have glaucous waxy surfaces, thick

epidermal cells with soluble and wall-bound UV-B screening metabolites (Laakso and Huttunen, 1998). Emerging *in vitro* shoot-tip needles may have been unable to regulate increased UV-B, an imbalance in photo oxidation implicated in oxidative stress in cell chloroplasts and mitochondria (Benson and Bremner, 2004). Other evidence of artificial environment induced stress in conifers relates to increased malondialdehyde (MDA) radicals, detected by electron paramagnetic resonance (EPR), in conifer needles of green–house grown plants compared to field-grown plants (Stegmann and Schuler, 2000).

The suppression of development in Sitka spruce was associated with Parafilm tube-sealing, whilst PVC cling-film showed well-formed mature embryo development (Selby, *et al.*, 1996a) . Silver nitrate or 2-chloroethylphosphonic acid (ethephon) in the culture medium indicated that ethylene was not responsible for this suppression. This study indicates the sensitivity of Sitka spruce in artificial culture and may explain cryopreservation recalcitrance, but further studies are required to indicate the physiological basis of the mechanism of the growth retardation seen in UAD cultures.

There was a strong family genotype difference in shoot-tip growth and family A showed double the accumulative extension of other families. Physiological studies were also undertaken on family differences as these may influence cryogenic tolerance. Strong genotype differences are common in shoot-tip culture maintenance, and have been noted in *P. sitchensis* previously (John and Murray, 1981). The shoot-tip families in this study share maternal genes, and these differences were observed as all members of family A showed greater growth vigour than the other four families cultured. Further studies would be required to confirm genetic predeterminism to vigour. Little work has been reported for genotype differences during maintenance of other plant species *in vitro*, although there are many reports indicating varied genotypic responses, from 100% success to complete recalcitrance, in initiation and rooting procedures (De Klerk, 2002).

4.4.2 Biochemical markers

There was no significant difference in genotype quenching of free radicals, but genotype AC showed double the amount of antioxidant quenching of genotype AA on a per mg protein basis (Fig.4.8c). Protein reduction and antioxidant increase are indicators that a plant is undergoing abiotic or biotic stress. This is the first time that antioxidant status has been reported in *in vitro* conifer shoot-tip cultures. In *Picea glauca* embryogenic suspensor cultures the redox state of glutathione was found to affect plant growth and cell proliferation. Inclusion of reduced glutathione promoted culture proliferation (Belmonte, *et al.*, 2003). Both

antioxidant and protein determinations are useful markers for *in vitro* culture health and prolonged maintenance and could be applied as a post-cryopreservation recovery assessment for Sitka spruce cultures.

No significant differences ($P>0.05$) were observed between UAD and NRS cultures in ethylene production. The plant hormone ethylene has been widely used as an indicator of plant stress (Benson and Withers, 1987). Studies have shown that ethylene evolution increases when plant tissues are subjected to wounding, pathogen attack or in tissue culture. The main pathway of ethylene biosynthesis in plant tissues is through 1-aminocyclopropane carboxylic acid (ACC) synthase, which converts S-adenosyl methionine (SAM) to ACC, the immediate precursor to ethylene. A second pathway is as a by-product of lipid peroxidation. However, the simple structure of ethylene means that many compounds could be converted to ethylene through various chemical reactions (Yang and Hoffman, 1984).

Greater ethylene production was observed in UAD cultures than NRS cultures, although their was not significant ($P>0.05$). Ethylene concentration in a sealed environment such as *in vitro* culture is reported to inhibit organised growth (Kumar, *et al.*, 1998). High ethylene content has been associated with reduced growth, shortening and thickening of stems in *in vitro* potato shoots (Biddington, 1992). However the fact that ethylene production was observed indicates that the membranes were still intact, as ethylene biosynthesis is dependent upon membrane integrity (Benson and Withers, 1987). In this experiment the source of ethylene evolution could not be determined. If ethylene production was via a lipid peroxidation pathway, ethane production may have been expected. The determination of accompanying lipid peroxidation products hydroxynonenal (HNE) and MDA would possibly clarify the ethylene pathway route. Stress analysis in *in vitro* cultured plants has also incorporated the analysis of methane evolution. Methane is produced when hydroxyl radicals are produced in the presence of DMSO, a free radical scavenger. In this study no methane evolution was observed thus supporting the possibility that ethylene was produced from hormone regulation.

There are no *in vitro* woody shoot-tip culture reports to compare ethylene responses. Ethylene was not determined to be responsible for somatic embryo maturation suppression in *P. sitchensis* (Selby, *et al.*, 1996a). In *Pinus sylvestris* callus ethylene production was detected during the first 2-3 weeks of culture and then decreased in line with growth reduction and increased browning (Lindfors, *et al.*, 1990). Ethylene evolution has been investigated in conifer needles, *Pinus ponderosa* and Jeffery pine and was found to significantly increase with age (Telewski, 1992). Conifer seedling growth response was found to be influenced by IAA and ethylene produced by ectomycorrhizal fungal symbionts (Scagel and Linderman,

1998). Further work is required in this area to clarify the relationship between *in vitro* cultured woody plants and ethylene production so that culture conditions may be further optimised. A time course, over several weeks, of volatile hydrocarbon sampling, with longer sealing times, would provide more information about the pattern of stress during each subculture and may assist cryopreservation protocol development.

4.4.3 DNA methylation

No significant differences ($P>0.05$) were observed between UAD and NRS cultures in DNA methylation. The methylation status of plants has been used as an indicator of stress (Benson and Bremner, 2004). Deoxycytosine is the most abundant nucleoside in plant organs and its content is usually 5-30% methylated. In Sitka spruce shoot-tip cultures the % methylation in deoxycytidine varied from 15.7 to 17.2%. There was a difference of 0.5% between UAD and NRS cultures. It is generally regarded that demethylation is associated with gene expression and methylation with gene inactivation. In comparison to other species the methylation and gene inactivation is low; pea, barley and corn cultures show greater (21-28%) methylation, of the deoxycytidine nucleoside (Klaas and Amasino, 1989) and in onion, pea, tobacco, rye and maize, 26.9 to 36.3% (Matassi, *et al.*, 1992). There are no reports of gymnosperm methylation levels. A lower methylation status may be a phylum characteristic, considering the earlier evolutionary requirements.

A high % RNA to DNA (ca 90%) was observed following extraction and may indicate active gene expression. However, this may be a consequence of the extraction technique and is being investigated by the UAD Plant Conservation Group (Johnston, *et al.*, 2006 In press). Another explanation is that total DNA has been reduced through successive 3-month subculture over 3.5 years of *in vitro* culture. In *Vitis* callus cultures a 10-fold decrease in observable DNA was recorded between callus induction to 1 year of *in vitro* culture (Harding *et al.*, 1996). Age related changes *in vitro* was also previously reported for conifer cultures (Bonga, 1989).

As only one species was used in this study the % G-C ratio was expected to be very similar throughout genotypes. The range between genotypes in this study was 41.6 to 43.1%; in other plant family nuclear genome investigations the GC levels covered a range from 35 to 47.7% (Matassi, *et al.*, 1992). There is evidence that DNA modifications are less stable in tissue-cultured than in seed-grown plants (Kaeppler and Phillips, 1993). However in Sitka spruce shoot-tip cultures the methylation status did not indicate signs of *in-vitro* induced stress. Methylation results were not measured over a period of time and it was not possible to

determine if physiological changes, such as 'dormancy' induced in different growth rooms could be mapped with methylation change as has been achieved in field-grown potato tuber meristems (Law and Suttle, 2003). This technique provided an insight into global DNA methylation changes but is not sensitive to specific subtle changes, as are restriction enzymes.

4.4.4 *In vitro* stress implications for cryopreservation

Blackening around the shoot-tip base was observed in both UAD and NRS cultures, but more predominantly in UAD cultures. It is hypothesised that high levels of phenolics may be responsible for low protein and high antioxidant activity. Phenolics may also interfere with protein extraction and mask the true content (Sakihama, *et al.,* 2002). In Sitka spruce shoot-tip cultures higher levels of ethylene were observed in UAD cultures than NRS. Ethylene is known to induce *de novo* synthesis of peroxidase and phenylalanine ammonia-lyase (PAL), an enzyme involved with lignification and phenylpropanoid synthesis of cells. Lignification of cells was confirmed in Scots pine callus by using a ß-glucosidase stain (Laukkanen, *et al.,* 1999).

The addition of poly-vinyl pyrrolidone (PVP) to woody plant tissue medium has been shown to remove phenolics. In *Ribes* and coffee (J.Johnston, pers. comm.) and in forest litter (Criquet, *et al.,* 2002) the addition of PVPP has also shown increased protein yield. Phenolic stress and lignification may lead directly to membrane damage and reduced ability of cultured shoots to survive osmotic stress and cryopreservation, and may generate other damaging secondary products of lipid peroxidation.

Shoot-tip cultures that are already stressed and manifesting free-radical driven damage may be less well equipped for stresses induced during cryogenic procedures. Necrotic tissue indicates membrane and cell wall damage, as H_2O exchange between the intracellular and extracellular compartments is vital if ice crystal formation is to be controlled or circumvented. It was because of the implications of *in vitro* stress that cultures were transferred from NRS to UAD within 6 months of cryopreservation experiments.

4.4.5 Pre-culture optimisation cold hardening

The response of *P. sitchensis* shoot-tips to continuous or alternating (8hr light at 20°C/16hr dark at low temperature, 4°C) cold treatments of 1-6 wks was investigated. A 1-week cold pre-culture in continuous darkness at 4°C was selected to induce the physiological and cellular changes associated with cold-hardening but without inducing a, 'physiological dormant,' stasis where time-consuming and unpredictable dormancy breaking treatments to re-induce regular development would have to be applied following cryopreservation. Shoot-

tips that had undergone this pre-culture phase showed a great improvement in desiccation tolerance compared to untreated explants; 30% of treated shoot-tips recovered after 6hr of laminar air-desiccation.

Cold treatment or conditioning in plants has been studied extensively and there is a wide variation in the requirements and response of *in vitro* shoot-tips to photoperiodic and temperature modifications. In tissue cultured *Rubus* sp. meristems 1 week of cold acclimation (25°C/-1°C/night temperature) preceding the excision of apical meristems improved post-cryopreservation survival from 18-41% in non-acclimated to 51-67% in acclimated meristems (Reed, 1988). It is essential to determine the optimal time of pre-culture and photoperiod regime as suboptimal conditioning such as over extensive pre-culture may result in decreased recovery performance (Kim, *et al.*, 2005). Following the optimisation of cold hardening at (1 week) the addition of pre-culture supplements (sugars, sugar alcohols and hormones) was also investigated.

4.4.6 Cold hardening and pre-culture supplements

It is thought that these pre-culture additives assist in priming the explant for cryopreservation by inducing the beneficial physiological changes of cold acclimation and by dehydrating tissues and stabilizing proteins and membranes during desiccation (Chang and Reed, 2000). *P. sitchensis* shoot-tip apices showed recovery following slow cooling to –10°C and for the first time, post-cryogenic recovery, after 1 week of cold hardening (4°C) and 9% (w/v) sucrose culture (Table 4.6). The order of pre-culture additive effectiveness in terms of *P. sitchensis* shoot-tip post-cryogenic survival after 28 days was sucrose (9% w/v), proline (5% w/v), ABA (10^{-4}M) and trehalose (9-11% w/v) and survival varied from 10 to 20% of shoot-tips cryopreserved.

Investigation through controlled rate cooling to –25°C provided continuous quantitative information on the effectiveness of pre-culture sucrose concentrations, whereas LN treatment indicated survival or no survival. The programmable freezer (Planar) also showed at what temperature ice nucleation occurred (approx.-8°C). This temperature corresponded well with the threshold temperature of shoot-tip recovery (-10°C) (Figs., 4.23 and Table 4.6). The cooling tolerance was heterogenous between replicates of the same pre-treatment. A similar method applied to *P. cordata in vitro* shoot-tips showed the most effective pre-culture, inducing cold hardiness to -22°C was a treatment of 150µM ABA followed by a 2 wk alternating low regime (Chang and Reed, 2001).

Sucrose and trehalose are both important in dehydration and freezing tolerance, but whereas sucrose has been routinely used for woody plant cultures in pre-cryopreservation culture [*V. vinifera* (Zhao, *et al.*, 2001), *P. cordata* (Chang and Reed, 2001), *R. nigrum* (Sherlock, *et al.*, 2005)], trehalose has not been so widely tested in higher plant culture. This may in part be due to its expense; and this is a limiting factor in it application for commercial genebanking. Its success as a cryo-protectant and apparent exceptional qualities are not fully understood, but thermal analysis indicates a high glass transition temperature associated with a weakened mobility of molecules within the bead matrix (Yoshii, *et al.*, 2000), superior effects in de-structuring the matrix network of water and the slowing down of matrix dynamics (Bordat, *et al.*, 2004) and its mechanism as both a proton donor and acceptor in forming hydrogen bonds with water allowing a superior hydration ability (Sakurai, *et al.*, 1997).

Post-LN recovery of *Betula pendula in vitro* shoot-tips with 40% recovery was achieved following 28 days of alternating cold hardening treatment (5°C) on medium containing 10^{-4}M ABA (Ryynanen, 1998). It is thought that an increase in ABA concentrations triggers expression of low-temperature genes (Chang and Reed, 2001). However, an unwanted consequence of its application can be an increase in callus formation during regeneration of cryopreserved shoot-tips (Ryynanen, 1998), and although this was not observed in *P. sitchensis* shoot-tips in this study, callus formation is a concern in clonal forestry germplasm storage where genetic preservation is critical. Proline has proven properties in desiccation stress resistance in higher plants and has high solubility, is neutral and non-toxic at high concentrations. It has been applied successfully to *Prunus* shoot-tips in cryogenic pre-culture at 2% (w/v) for 24hr (Brison, *et al.*, 1995). After 3 months the surviving *P. sitchensis* shoot-tips showed signs of degradation possibly because of wounding injury induced during dissection or suboptimal post–culture, these factors were considered in further investigations.

4.4.7 Dissection injury

Dissection injury may further compromise cryogenic recovery as free radicals generated from the wounding response, are more readily able to react with cryo-damaged membrane lipids, perpetuating lipid peroxidation and hydroxyl radical formation. DMSO is a scavenger of hydroxyl radicals as well as a cryoprotectant and a short post-dissection incubation was applied to *P. sitchensis* shoot-tip apices to ameliorate the damage caused through dissection (Benson, *et al.*, 2006 In press). A 24hr 5% (v/v) DMSO filter bridge incubation of shoot-tips was determined to be the most successful post-dissection treatment for this study (Fig. 4.16).

4.4.8 Explant optimisation and preliminary cryopreservation tests

Following the characterisation of cultures and pre-culture optimisation, cryopreservation trials were implemented to select the optimal explant size and development type, desiccation, pre-treatment regime, cooling method and post-cryogenic recovery culture. Initially active apical meristems and dormant lateral buds were dissected, encapsulated, osmotically dehydrated, desiccated and immersed in LN, without a pre-treatment regime. Survival at each stage was considered and although no shoot-tip apices survived following the desiccation or LN immersion procedures, indicators of protocol refinements established that smaller dissection sizes were optimal for desiccation treatment and that apical meristems were more suitable than lateral buds because of the physiological production consistency.

The selection of tissues from the shoot apex region may be necessary for cryopreservation (Benson, *et al.*, 2006 In press). The shoot apex meristematic dome produces cells that will be incorporated into axillary meristems, inflorescence organs and stem tissue (Carles and Fletcher, 2003). The selected tissues may include the apical dome, unexpanded needle primordia, the crown through which the meristem is attached to the shoot and in the case of dormant buds the protective primordial casing that has evolved to shield the meristematic dome from the desiccation and freezing stresses of boreal, temperate extreme winters. In the case of conifers, selection of tissue may be particularly important because work on field-grown dormant buds has shown that certain regions of the shoot apex region act as ice-sinks to control ice formation and limit its presence in the most vital developmental tissue the apical dome (Sakai, 1983). In this study a dissected explant where all tissue was removed except the apical dome and immature primordia and the length of the dissected tissue was 1-3mm top to base, showed the best survival following encapsulation and dehydration. Tissue tolerance to dehydration and desiccation procedures may be linked to the smaller number of vacuoles observed in the immature, tissue compared to mature needles. Thermal analysis through Differential Scanning Calorimetry (Chapter 5) may provide more detail on the tissue vulnerabilities that permit detrimental ice crystal formation.

Research in *Prunus* rootstock has indicated that there is a correlation between shoot-tip meristem range and post-cryopreservation recovery (Brison, *et al.*, 1995). Total % shoot-tip post-LN recovery was 6.5% when meristems were dissected to 0.3-0.5mm and 70% when they were dissected to 1.5-2mm. Recovery of post–cryogenic garlic meristems was also optimal in shoot-tips sized 3mm diameter x 3mm height (including a 1.0mm basal plate) (Kim, *et al.*, 2005).

In this study, untreated lateral bud meristems survived encapsulation, dehydration and desiccation, and LN immersion whereas active apical meristems did not (Fig. 4.19). Post-LN recovery of field collected dormant shoot-tips has been achieved in several species of woody plants including apple (Katano, *et al.,* 1983) and mulberry (Yakuwa and Oka, 1988). Recovery was also observed from partly dormant *Pinus sylvestris* field collected buds following -80°C storage, but only when buds were collected at certain times of year January and April, (Kuoksa and Hohtola, 1991). It is known that physiological, cellular and cytoplasmic changes accompany the formation of dormant buds that enable evergreen conifer trees to survive the cold and desiccation of winter (Havranek and Tranquillini, 1995). Attempts to induce low temperature hardiness and dormancy in *in vitro* Saskatoon berries comparable to fully hardened field grown buds were unsuccessful (Baldwin, *et al.,* 1998).

Axillary buds sampled from *in vitro* plants of *Vitis vinifera* showed a maximum of 40.9 % re-growth following a pre-treatment regime of 4 months without subculture + 1 month cold-hardening at 5°C under a modified photoperiod. Without this procedure no re-growth was achieved. Such a lengthy procedure would not be feasible for a large-scale genebanker working with hundreds of genotypes. Another factor in the application of this technique to a multi-clonal genebanking programme is the genotype variability and subculture age inconsistency of lateral shoots. Decreased production of axillary shoots over subculturing time has been demonstrated in *P. sitchensis* previously (John and Murray, 1981).The incorporation of lateral bud meristems within the *P. sitchensis* cryopreservation programme was excluded because of the inconsistent production and developmental stage of *in vitro* lateral buds resulting in heterogenous re-growth capacity.

Investigations into the response of shoot cultures and alginate encapsulation was undertaken on *in vitro* shoot cultivars of *Camellia japonica* and *Camellia reticulate* (Ballester, *et al.,* 1997) and encapsulated 5-6mm shoot-tips showed a similar or significantly better propagating rate than non-encapsulated controls. Other post-cryopreservation encapsulation based studies have maintained the post-cryogenic shoot explant within the matrix, until it grew clear of the matrix (Hirai and Sakai, 1999, Reed, 1988).

4.4.9 Dehydration and desiccation parameters

Results indicated that encapsulated apical meristems survived the osmotic dehydration procedure but showed limited recovery following the laminar air-flow desiccation procedure. Initially a 3hr desiccation period was applied; however % RM determination (Fig. 4.21a) and survival (4.21b) indicated that a 4hr desiccation time would produce a more suitable bead

moisture content (20-25%) for vitrification during cryopreservation. This investigation also showed that 0.75M sucrose osmotic dehydration (18hr) was important to ensure the rate of water loss of the encapsulated shoot-tips, during the preceding laminar air desiccation phase, was not so rapid that the cells were irrecoverably altered.

Other woody plant encapsulation-dehydration research indicated similar desiccation requirements of 4hr 20-25% RM such as *Ribes* (Benson, *et al.*, 1996, Reed, 1990). Encapsulated–dehydrated shoot-tips of *V. vinifera* were reported to require 8-10hr of laminar air-flow desiccation to produce a water content of 15.6 and 17.6%, but these beads were supplemented with 2M glycerol and 0.4M sucrose in the alginate matrix. Survival plots for *P. sitchensis* showed that even after 2hr of desiccation no untreated shoot-tips recovered whereas in *V. vinifera* an equivalent desiccation time showed survival as high as 85%. A pre-culture or pre-treatment phase was required to prime the *P. sitchensis* shoot-tips for desiccation and cryopreservation.

4.4.10 Encapsulation-vitrification

An encapsulation-vitrification method was selected for testing on *P. sitchensis* shoot-tips because during encapsulation-dehydration, recovery was noted to fall following dehydration treatments. Encapsulation-vitrification requires a shortened dehydration process. In several cases it has resulted in a higher rate of survival and a faster recovery growth for example in *Wasabia japonica* (Matsumoto, *et al.*, 1995) and *Solanum* spp. (Hirai and Sakai, 1999). The encapsulation-vitrification process for cryopreserving meristems involves a preculture and /or loading phase using high concentrations of sucrose and glycerol followed by short exposure to PVS2, a highly concentrated mixture of cryoprotectants. These conditions enable vitrification and the mitigation of ice crystal or plasmolysis injury.

P. sitchensis pre-cultured shoot-tip meristems showed the greatest recovery (85%) following the application of a loading solution and PVS2 at 0°C for 30 min, although no shoot-tips recovered following LN–immersion. The PVS2 treated meristems showed a greater recovery than meristems treated only with loading solution, although the difference between the PVS2 replicates was greater (60%). This may be because of the cell stimulating and elongation properties of DMSO that will be re-visited in Chapter 7. When shoot-tips were exposed to longer PVS2 treatments, recovery was reduced to 30-60%. The pre-conditioning, 'loading solution,' step is important to osmoprotect the meristems from harmful osmotic stress, and was essential for the cryopreservation of *Wasabi* (Matsumoto, *et al.*, 1995).

This method has been shown to be effective for the cryopreservation of woody species. *Ribes* shoot-tip meristems, cryopreserved using encapsulation-vitrification showed recovery exceeding conventional cooling methods (slow cooling) in terms of recovery growth and shoot formation (Benson, *et al.*, 1996). In commercial application the encapsulation-vitrification procedure is more time efficient where no extended laminar air-flow desiccation time (2-6hr) or facilities are required. Uniform, newly initiated shoot-tip cultures that had not undergone several sub-cultures may show an improved post-LN encapsulation-vitrification recovery response and it seems this method is more suited to these desiccation sensitive explants.

4.4.11 Post-cryopreservation treatment

A preliminary investigation was undertaken to optimise the post-cryopreservation culture of *P. sitchensis* encapsulated shoot-tip apices. It was previously determined that shoot-tips showed better survival when they were not removed from their Na-alginate bead. PGRs (kinetin, gibberelic acid, and thiadurazon) were incorporated because shoot-tips were initially recovering and then dying in post-treatment culture. *In vitro Anigozanthus viridis* shoots showed improved post-cryogenic recovery following culture in a post-cryopreservation medium containing a combination of 0.5M cytokinin (kinetin or zeatin) and 0.5M GA_3 (Turner, *et al.*, 2001b).

In this study, growth of pre-cultured dissected meristems of *P. sitchensis* percentage growth appeared to be inhibited by the 14 day PGR treatment. While recovery of the control cultures was between 80 and 100%, those shoot-tips treated with 2 weeks of PGRs showed varied recovery between 5% (1μM GA_3) and 80% (0.5μM TDZ). Turner, *et al.* (2001b) reported that all PGRs applied (1μM zeatin; 0.5μM kinetin; 0.5μM kinetin and 0.5μM GA_3; 0.5μM kinetin + 0.5μM GA_3 +0.5μM IAA) for 3 weeks, produced high levels of post-LN recovery in *Anizoganthos viridis* shoot-tips and were only slightly lower when cytokinin was excluded. This research also highlighted the importance of applying the PGRs within the first 7 days of recovery.

Further experiments to determine the effect of PGRs following LN immersion were not undertaken because of the results of the preliminary examination where PGRs appeared to be inhibiting growth in *P. sitchensis* shoots. More work is required to optimise post-cryogenic recovery in conifer shoot-tip cultures. The physiological basis for the growth inhibition is not understood as cytokinins and gibberellins are associated with plant cell stimulation and elongation, apical dominance stimulation, senescence prevention and dormancy breaking (Raven, *et al.*, 1999).

Post-cryopreservation shoot-tips are vulnerable both to physical handling and increased free-radical attack and in particular phenolic oxidation, from from desiccated, freeze damaged necrotic tissue.

4.4.12 Future recommendations

It is recommended that younger, recently initiated shoot-tip cultures (> 6months from germination/initiation) may show greater cryo-tolerance and survival without the accrued damage of long-term culture. Shoot-tip cultures were 5 years old by the end of study and showed a browning stain in agar that could be a product of phenolic oxidation. Intra-genetic developmental heterogeneity may be reduced in younger cultures. A test for phenolic oxidation may be applied over successive subcultures to determine at what level phenolic accumulation is critically affecting development. Currently, further *in vitro* stabilisation and an understanding of *in vivo* physiology in the *in vitro* environment is required to enable shoot-tip encapsulation-based cryopreservation protocols to become a feasible genebanking storage method for *P. sitchensis*.

4.5 Conclusions

P. sitchensis in vitro shoot-tip cultures are highly recalcitrant, showing broad developmental and physiological variation between genotypes and within genotypes resulting from: (1) *in vivo* generated growth problems manifested because of extreme ecophysiological adaptations of the donor plant often resulting in dormancy and growth flush patterns and (2) a sensitivity to micro-environmental changes.

Progress has been achieved in developing pre-established biochemical tools for *P. sitchensis* shoot-tips, to better characterise the shoot-tip cultures and assist in ameliorating environmental stress, these techniques may also be used to assist in the development of cryopreservation protocols. Oxidative stress and DNA methylation patterns indicated no significant differences between genotypes of the same family or genotypes tested following 6 months culture at different institutions (UAD and NRS). Temporary shoot-tip apex survival and re-growth was achieved following 1 wk of 4°C cold acclimation in 9% (w/v) sucrose, 4hr laminar air-flow desiccation and LN immersion, but degradation and senescence occurred after 3 months. A range of PGRs at 0.5 and 1μM did not improve growth when compared to untreated controls. In trials without LN treatment, encapsulation-vitrification showed greater recovery than

encapsulation-dehydration indicating the desiccation sensitivity of the shoot-tip apices. The physical cryogenic factors involved in shoot-tip apex recalcitrance will be investigated through thermal analysis in Chapter 5.

Chapter 5 THERMAL ANALYSIS OF SHOOT-TIP APICES, SOMATIC EMBRYOS AND EMBRYOGENIC SUSPENSOR MASSES

5.1 Introduction

The aim of this chapter is to study and profile the biophysical responses of a range of different *in vitro P. sitchensis* cultures to different cryopreservation protocols. Differential scanning calorimetry (DSC) will investigate the physical state of water in cryopreservation protocols which have previously been applied to different explant types of *P. sitchensis* including: embryogenic suspensor masses, ESM (Chapter 2), somatic embryos (Chapter 3) and shoot-tip apices (Chapter 4). Further objectives are to gain a better understanding of cryoprotectant efficacy with a view to optimising vitrification–based cryopreservation methods and to increase the fundamental knowledge base of Sitka spruce cryogenics.

5.1.1 Applications of differential scanning calorimetry

Differential Scanning Calorimetry is used to analyse the physical state of water during cooling and re-warming of samples by measuring the heat-flow during a preset (10°C/min) cool and warming cycle. The instrument compares heat flow between two pans the sample tissue is sealed in an aluminium pan and compared with a control reference pan and the thermal difference (mWatts) is plotted against time, or temperature, to produce a thermogram (Benson, *et al.*, 2005 In press). Ice formation (nucleation), re-crystallisation, melting, de-vitrification and glass formation/relaxation all produce thermal events that alter the heat input required to maintain both pans at the same temperature. This causes a change in heat, manifested as an exothermic, or endothermic event, resulting from the latent heat of fusion or melting of water. Thermograms during cooling can show: (1) an exothermic peak for ice nucleation and (2) step-like deflections from the baseline during the transition from liquid to amorphous glass transitions (Tgs). Thermograms during re-warming can show: (1) an endothermic peak associated with melting, and (2) step-like deflections from the baseline during glass relaxation transitions (Tgs). The thermograms produce information about the water status in samples and how effective vitrification treatments are in reducing ice formation during a cryopreservation protocols. Therefore, understanding the thermal properties of cryoprotective agents can help develop and optimise cryopreservation procedures.

5.1.1.1 Osmotically active (OA) and inactive water (OI)

In biological cells there is a water component present that does not form ice in a supercooled state (Benson, *et al.*, 2005 In press). This water component is referred to in the literature as unfreezable water, unfrozen water, bound water and osmotically inactive water. In this study the water component will be termed as either osmotically inactive or osmotically active. Osmotically active water is determined using the enthalpy melt constant for water and the area of the melt endotherm peak (Block, 2003). The osmotically inactive component is calculated as the difference between total water and osmotically active water.

Mazur (2004) reviewed a number of nuclear magnetic resonance (NMR) and DSC– based methods and established that in the various cell types investigated, 0.2 to 0.3g water per gram dry weight or 10% of water in fully hydrated cells that does not exhibit heat of crystallization or melting. The removal of this water has been shown to be detrimental, with consequences including disruption in macromolecule confirmation, interference with enzymatic reactions and lipid bilayer membrane fusion (Mazur, 2004). Sun, (1999) determined damage to acorns, using electrolyte leakage as the criterion, was increased sharply when the water content was reduced below 0.3g per g DW. This study will examine the thermal profiles and water status in three different explant types of Sitka spruce tissue cultures.

5.1.2 Thermal analysis of shoot-tip apices

The thermal status of shoot-tip apices was investigated in order to assist future encapsulation-dehydration and encapsulation-vitrification (PVS2) cryopreservation protocol optimisation (Chapter 4). Previous studies showed little survival and no recovery following cooling and the objective of this investigation was to determine if there is a thermodynamic explanation for Sitka spruce shoot-tip recalcitrance. Current literature indicates that there have been no previous thermal investigations on *in vitro* explants of conifer species. Thermal analysis was applied to *in situ* lateral buds of Douglas-fir (*Pseudotsuga menziesii*) seedlings, grown under non-hardy and hardy conditions, and was used to detect low-temperature exotherms and the nucleation of supercooled aqueous fractions, indicative of lethal freezing (Stushnoff, *et al.*, 1992).

Thermal analysis was first applied to encapsulated cryopreserved plant tissue by Dereuddre, *et al.*, (1991). Alginate-coated somatic embryos of carrot were examined under desiccation and freezing treatments. The physical/chemical explanations for these biological transitions were interpreted from the fundamental work of Mazur, (1983) and Meryman and Williams

(1984), see Chapter 1. To date, thermal analysis has been applied to the shoot-tip cultures of several woody plant species; through encapsulation-dehydration *Ribes ciliatum* (Dumet, *et al.*, 2000, Sherlock, *et al.*, 2005), olives (Martinez, *et al.*, 1999) and hops (Martinez and Revilla, 1998) and vitrification through PVS2 in *R. nigrum* (Benson, *et al.*, 1996). Chemical vitrification (usually by PVS2 or PVS3) of shoot-tip cultures has been applied to naked shoot-tips/meristems of at least four species *Dianthus caryophyllus* L. (Tannoury, *et al.*, 1991), sugarcane (Gonzalez-Arnao, *et al.*, 1996), *Ribes* (Benson, *et al*, 1996) and garlic (Kim, *et al*, 2005).

5.1.3 Thermal analysis of somatic embryos

Encapsulation-dehydration and PVS2-based vitrification of isolated naked somatic embryos was undertaken (see Chapter 3). Both protocols produced up to 100% post-LN recovery of non-embryogenic masses, and thermal analysis was therefore applied to determine critical points of evaporative laminar air-desiccation and PVS2-vitrification.

There are limited reports of experimental investigations in this area. Aside from preliminary work on encapsulated carrot somatic embryos (Dereuddre, *et al.*, 1991), thermal analysis has been applied to some woody species somatic embryos including naked desiccated (laminar air-flow) embryos of oil palm (Dumet, *et al.*, 1993), walnut (Deboucaud, *et al.*, 1994) and encapsulated-dehydrated Neem (Benson, *et al.*, 2005 In press). There are no reports to date of thermal analysis being undertaken following PVS2 treatment on isolated naked somatic embryos.

5.1.4 Thermal analysis of embryogenic suspensor masses

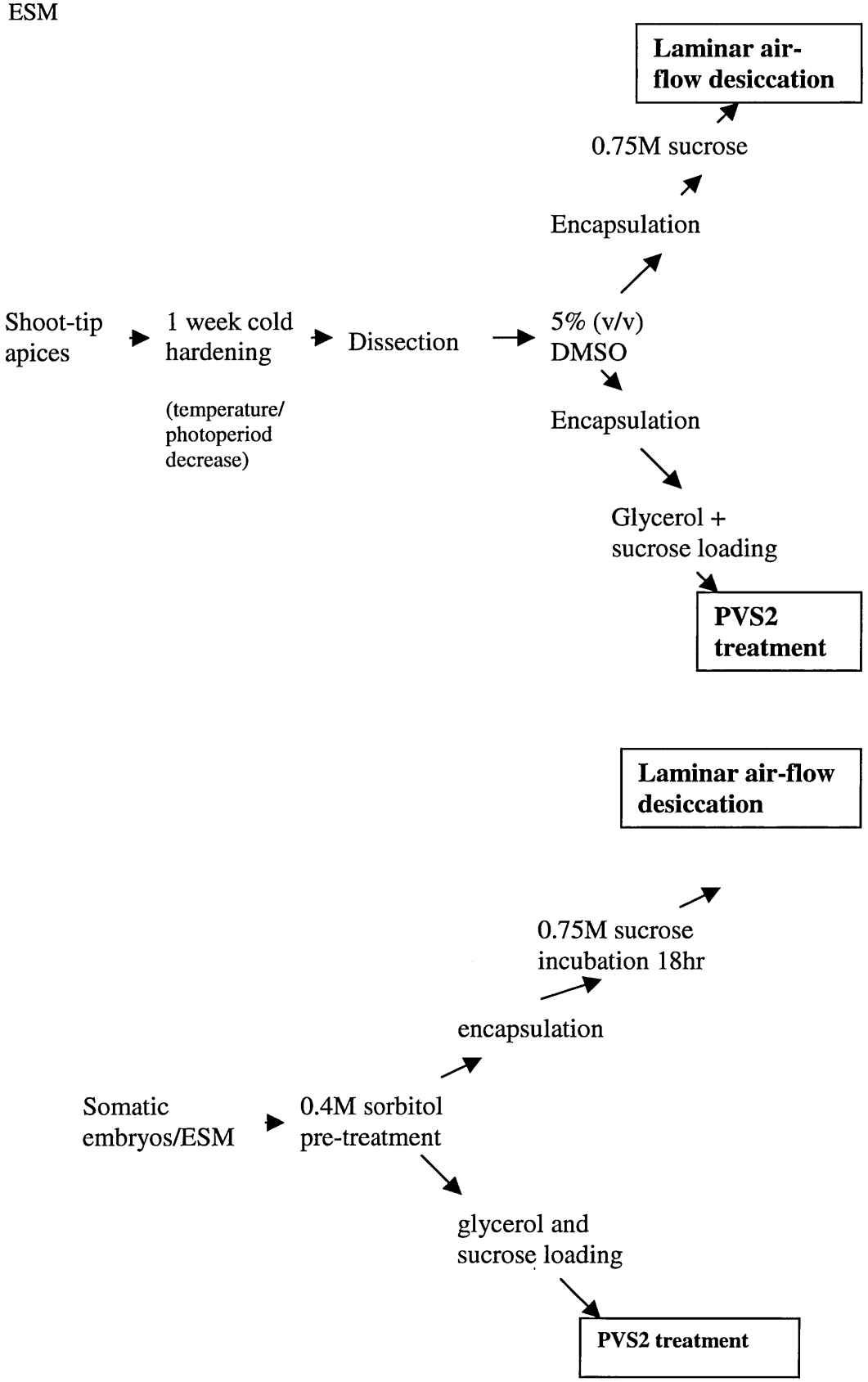
An encapsulation-dehydration protocol developed for ESM (Chapter 2) will be investigated by thermal analysis. Limited (25%) post-LN encapsulated ESM recovery was observed in one genotype only, whereas greater (20-100%) post–LN recovery was observed for 5 genotypes when they were cryopreserved using other methods (controlled rate cooling). DSC was applied to determine if the encapsulated-dehydrated ESM was sufficiently desiccated to circumvent ice nucleation and if a stable glass was formed. Thermal analysis has been previously applied in encapsulation-dehydrated suspension cells of *Arabadopsis thaliana* (Bachiri, *et al.*, 2000) and *Catharanthus roseus* (Bachiri, *et al.*, 1995), and in algal cryopreservation, using vitrification, (Harding, *et al.*, 2004).

5.2 Materials and Methods

5.2.1 Experimental strategy

This study will investigate thermal analyses for 3 explants; shoot-tip apices, somatic embryos and embryogenic suspensor masses following three tested cryopreservation protocols: encapsulation-dehydration, encapsulation-vitrification and vitrification. Explant treatments prior to cooling/warming cycles during DSC analysis are shown in Figure 5.1. Table 5.1a and 5.1b show the specific combinations of treatments to determine the optimum treatment for cooling/warming, and examine the effects of individual treatments. The pre-treatment regimes for each explant type were optimised and discussed in previous experiments (Chapter 2 for ESM, Chapter 3 for somatic embryos and Chapter 4 for shoot-tips). All DSC analyses will be undertaken within 3 months, in an air-conditioned laboratory at 20°C $\pm$ 2.

Figures 5.1. Pre-treatment methods for Sitka spruce shoot-tip apices, somatic embryos and ESM



PVS2= Plant Vitrification Solution 2
ESM =Embryogenic suspensor masses

Table 5.1a Shoot-tip apices treatments investigations

Pre-treatment	Method of cryopreservation	Method of encapsulation	Pre-treatment	Time (hr) pre-cryopreservation treatment
ch +DM	Encapsulation-dehydration	Na-alginate in MS	None	None
ch +DM	Encapsulation-dehydration	Na-alginate in MS	0.75M sucrose	18hr
ch +DM	Encapsulation-dehydration	Na-alginate in MS	Laminar air-flow	3hr
ch +DM	Encapsulation-dehydration	Na-alginate in MS	0.75M sucrose /laminar air-flow	18hr/3hr
ch	Encapsulation-dehydration	Na-alginate in MS	0.75M sucrose /laminar air-flow	18hr/3hr
DM	Encapsulation-dehydration	Na-alginate in MS	0.75M sucrose /laminar air-flow	18hr/3hr
ch +DM	Encapsulation-dehydration	Na-alginate in MS	0.75M sucrose /laminar air-flow	18hr/4hr
ch +DM	Encapsulation-vitrification	Na-alginate +sucrose (MS)	None	None
ch +DM	Encapsulation-vitrification	Na-alginate +sucrose (MS)	Glycerol+sucrose at 0°C	2hr
ch +DM	Encapsulation-vitrification	Na-alginate +sucrose (MS)	Glycerol+sucrose at 0°C +PVS2	2hr/1hr
ch +DM	Encapsulation-vitrification	Na-alginate +sucrose (MS)	Glycerol+sucrose at 0°C +PVS2	2hr/2hr

Legend:

*Blank Na-alginate beads also tested STA= Shoot-tip apices

ch=Cold Hardening (1 wk) alternating 8hr under fluorescent tubes (20 ± 1 °C), 16hr dark (4± 2°C)

DM= 5% (v/v) DMSO (12hr) prepared in liquid MS (Murashige and Skoog) media.

0.75M sucrose in liquid MS media incubation on rotary shaker at 125rpm for 18hr at (20 ± 1 °C).

2M glycerol and 0.4M sucrose in liquid MS. PVS2 =Plant Vitrification Solution 2 in liquid MS

Table 5.1b Embryogenic suspensor masses and somatic embryos treatment investigations

Explant*	Pre-treatment	Method of Cryopreservation	Method of Encapsulation	Pre-treatment	Time (hr) pre-cryopreservation treatment
Somatic embryo	0.4M sorbitol	Encapsulation-dehydration	Na-alginate in SEM	0.75M sucrose	18hr
Somatic embryo	0.4M sorbitol	Encapsulation-dehydration	Na-alginate in SEM	0.75M sucrose/ laminar air-flow	18hr/1hr
Somatic embryo	0.4M sorbitol	Encapsulation-dehydration	Na-alginate in SEM	0.75M sucrose/ laminar air-flow	18hr/2hr
Somatic embryo	0.4M sorbitol	Encapsulation-dehydration	Na-alginate in SEM	0.75M sucrose /laminar air-flow	18hr/3hr
ESM	0.4M sorbitol	Encapsulation-dehydration	Na-alginate in SEM	0.75M sucrose /laminar air-flow	18hr/3hr
ESM	0.4M sorbitol	Encapsulation-dehydration	Na-alginate in SEM	0.75M sucrose /laminar air-flow	18hr/4hr
Somatic embryo	0.4M sorbitol	Chemical Vitrification	None	None	None
Somatic embryo	0.4M sorbitol	Chemical Vitrification	None	PVS2	0.5hr
Somatic embryo	0.4M sorbitol	Chemical Vitrification	None	PVS2	1hr

*Blank Na-alginate beads also tested for combinations ESM - Embryogenic suspensor masses

Culture on 0.4M sorbitol in solid SEIM media (somatic embryo) and SEM media (ESM) for 48hr in dark

0.75M sucrose in liquid SEIM/SEM media incubation on rotary shaker at 125rpm for 18hr at (20 $\pm$ 1 °C) in dark.

PVS2 =Plant Vitrification Solution 2 prepared with liquid SEIM

5.2.2 Shoot-tip apices

Shoot-tip apices were excised, cultured and pre-treated as discussed in Chapter 4, and shown in Fig. 5.1a, with the following combinations: 1 week cold hardening in an alternating light/dark, 20°C/4 °C; and 12hr incubation on sterile filter paper soaked in 5% (v/v) DMSO in liquid MS. Shoot-tip apices cryopreserved by encapsulation-dehydration were encapsulated in (3% w/v) Na-alginate followed by 0.75M sucrose osmotic dehydration for 18hr (prepared in liquid MS) on a rotary shaker at 20°C and were then desiccated in a laminar air-flow for 0-4hr, (Chapter 4). Shoot-tip apices cryopreserved by chemical vitrification (PVS2) were encapsulated in 2% (w/v) Na-alginate supplemented with 0.4M sucrose; incubated in 2M glycerol and 0.4M sucrose for 2hr at 0°C, and then incubated in Plant Vitrification Solution 2 (PVS2) for 0-2hr.

5.2.3 Somatic embryos and embryogenic suspensor masses

Somatic embryos (size 2-3) and ESM were induced (Chapter 2) and pre-treated with 0.4M sorbitol in SEM media (ESM) and SEIM media (somatic embryos) for 48hr as shown in Fig. 5.1b. ESM and somatic embryos selected for encapsulation-dehydration were encapsulated in 5% (ESM) or 3% (somatic embryos) (w/v) Na-alginate prepared with SEMM/SEIM liquid media, incubated in 0.75M sucrose (prepared in liquid SEMM/SEIM) for 18hr on a rotary shaker at 20°C, and then desiccated in a laminar air-flow cabinet for 0-4hr. Somatic embryos selected for PVS2 vitrification were osmoprotected in a loading solution containing 2M glycerol and 0.4M sucrose (prepared in liquid SEIM) for 60 min at 0 °C and were then vitrified in PVS2 (prepared in liquid SEIM) at 0°C for 0-2hr (as described in Chapter 3 section 3.2.4.2).

5.2.4 Alginate bead preparation

Specific alginate bead and treatment combinations tested are shown in Tables 5.1a and b. The fresh weight of beads was determined on a micro-balance to an accuracy of 4 decimals (PAG OERLIKON AG Zurich) following each treatment, then were transferred to 50µl aluminium pans and sealed using a Perkin-Elmer pan crimper. Beads that had not been desiccated or had only been laminar-air desiccated for 1-2hr were cut into sections to fit the DSC pan; the explant or a section of the explant was included in this bead section. Beads that had been laminar-air desiccated for 2hr or more were not sectioned, as they were a sufficiently small to fit the DSC pan. The fresh weight of the bead and pan was recorded prior to transfer to the

DSC cooling block. The pan and contents were transferred, following DSC analysis, to an oven and dried for 12hr at 105°C. The dry weight of the pan and bead was recorded.

Assessment of Moisture Content Status

The moisture content of the beads was determined from the fresh weight (FW) of a sample of beads (where n = 2-3 replicate beads per treatment) from each stage in the alginate bead encapsulation procedure, 2-step osmotic dehydration in sucrose and laminar air-flow desiccation. These bead samples were dried over silica gel at 105°C overnight to determine their dry weight (DW). The % residual moisture (%RM) per bead was calculated from these parameters as (FW – DW)/FW x 100 = %RM. Rates of desiccation, % RM profiles per treatment and the freezable water content of the sample (g/g DW) were calculated from means of these values.

5.2.5 Differential scanning calorimetry

DSC was performed following laminar air-flow desiccation. DSC was undertaken as described by Benson, *et al.*, (1996) using a Perkin Elmer DSC 7, with Pyris 7 software, calibrated using zinc, indium and pure water as standards.

5.2.6 Data analysis

Data from each cryopreservation experiment comprises a mean ± SEM of 2-3 cryovials. DSC data are presented in three ways: (a) as means ± SEM of ice nucleation/glass transition (Tg) and melt/Tg events, (b) the mean ± SEM of osmotically active and inactive water, calculated by using the melt endotherm, based on the enthalpy of water value (334.5 J/g) (Block, 2003) together with the total water content of the sample :

% OA water = OA/TW x100
% OI water= (TW-OA)/TW x100
OA g/g/DW=OA/B
OI g/g/DW= (TW-OA)/B

Where:

$$OA = \frac{\text{Melt peak area}}{334.5}$$

TW= Total bead water content (mg) determined from bead total fresh weight- total dry weight

B= Bead weight (mg) dry weight

Osmotically inactive (OI) is unfrozen; osmotically active (OA) water is frozen.

(c) Representative thermal profiles (Excel graphics) were presented for each treatment/explant. DSC profiles were produced by the Pyris 7 software using a scanning rate of 10±°C/min. Scans were performed from 25°C to -150°C for cooling and -150°C to 25°C for heating profiles. Pyris 7 compiled data, was transformed graphically by Excel (MS 2000) to the figures shown.

5.3 Results

5.3.1 Shoot-tip apices

5.3.1.1 Encapsulation-dehydration

Shoot-tip apices were excised from *in vitro* cultures and pre-treated with different combinations of sucrose (suc), DMSO (DM) and cold hardening (ch) before laminar air-flow desiccation (0-4hr). DSC analysis (Table 5.2) was undertaken and the data are presented in three ways: as changes in water content (% RM), representative examples of cooling and warming profiles (Figs 5.2 and 5.3) and summaries of thermal characteristics (Tables 5.6 and 5.7). Ice nucleation/melt events were successfully eliminated and replaced by Tg events when beads incorporating shoot-tip apices (st) were pre-treated with DMSO, sucrose and desiccated for 3hr (Tables 5.6 and 5.7). Sucrose, DMSO and cold hardening pre-treatments had a critical effect on the mean % water content (fresh weight, FW and dry weight, DW) of encapsulated shoot-tip apices after 3hr desiccation (Table 5.2). Control blank (blk) Na-alginate beads were also compared.

Table 5.2 shows the bead weight range (% RM), from the smallest 6.5mg (st-suc+ch+DM) to the largest 40mg (st+suc+ch+DM). Beads that did not undergo desiccation were cut to size in order to fit into the aluminium DSC pan. After 3hr of laminar air-flow desiccation it was not necessary to reduce the bead size. The greatest variation range in bead size between replicates in the 3hr-desiccated beads was in the treatment group (st-suc+ch+DM) where the beads had not been dehydrated with sucrose and a bead weight variance of 12.5mg. The smallest difference in bead size between replicates in the 3hr desiccated beads was in the treatment group (st+suc+DM-ch) where the shoot-tip apices had not been cold hardened and the bead weight difference was 0.1mg. Within each treatment group the replicate bead with the highest fresh weight produced the largest osmotically active water (OA) water values.

The mean % water content (FW) ranged from 72.1% (blank bead no treatments) to 31.0% (st+suc-DM+3hr) (Table 5.2). The OA content was reduced from 2.12±0.11 g/g DW to 0 g/g

DW following sucrose dehydration and air-flow desiccation (0-4hr). The osmotically inactive water (OI) content fluctuated depending on the pre-treatment regime, but using the example of an encapsulated shoot-tip apice with all pre-treatments (st+suc+ch+DM) at 0hr of desiccation the OI was 0.73± 0.3g/g DW, this increased to 1.04±0.23 g/g DW after 3hr desiccation and dropped to 0.893±0.05 g/g DW after 4hr desiccation.

The mean % water content of beads (st+suc+ch+DM+3hr) was compared with beads incorporating shoot-tip apices (a) without DMSO treatment, (b) without cold hardening and (c) without sucrose pre-treatment. When DMSO pre-treatment was excluded the mean % water content on a fresh weight basis was ca. 20% less. When sucrose incubation or cold hardening was excluded from the pre-treatment programme the mean % water content was 10% greater.

Ice nucleation/melt peaks were formed in all beads that had not undergone sucrose osmotic dehydration (Fig. 5.3, Tables 5.6 and 5.7) with or without 3hr laminar-air desiccation. When beads were osmotically dehydrated in sucrose and desiccated for 3hr no ice nucleation/melt events were observed and glass transitions (Tg) occurred (Fig 5.3c and d). Sucrose-osmotically dehydrated beads followed by 4hr desiccation, showed no freezing, melt or glass transition events.

Figure 5.2 and Table 5.6 show that the blank bead without sucrose osmotic dehydration and no desiccation produces ice nucleation (-255.7±5.3 J/g) and the melt event is double that of the height of the corresponding peak in a bead under the same conditions containing a shoot-tip apex (-169.4 ± 26 J/g). Table 5.7 shows that following 3hr desiccation, sucrose dehydration, blank beads produced melt peaks (OA water presence), but beads incorporating shoot-tip apices did not. Ice nucleation events fluctuated according to bead size and treatment but generally occurred at -15°C and ended at -25°C (Table 5.6), ice melt events occurred at 0°C and ended between 5 and 21°C (Table 5.7). The enthalpy of these peaks decreased through progressive desiccation. Glass transition (Tg) events, also fluctuated according to bead size and treatment; cooling onset from -49 to -108.5°C; Tg events on warming onset from -58.5 to -19.7 °C.

5.3.1.2 Plant Vitrification Solution 2

Cold hardened shoot-tip apices were excised from *in vitro* cultures and pre-treated with 5% (v/v) DMSO, encapsulated in Na-alginate incorporating 0.4M sucrose (prepared in MS media), osmoprotected in 2M glycerol and 0.4M sucrose (osmoprotection treatment referred

to as 'gly' in tables) for 2hr at 0°C and treated with PVS2 (PV) for 30min to 2hr at 0°C. DSC analysis was undertaken and the results are shown in three ways: (1) the change in water content (Table 5.3), representative examples of cooling and warming profiles (Fig. 5.4), and summaries of the mean onsets, midpoints and ends of thermal events and the associated enthalpy/heat capacity (Tables 5.8 and 5.9). Ice nucleation/melt events and Tgs were not observed after shoot-tip apices had undergone all treatment with 2hr PVS2 incubation (Tables 5.8 and 5.9) but were observed at earlier stages in the protocol. The mean % FW water content remained at 71-96% throughout increasing dehydration and cryoprotection treatments but the proportion of osmotically active (OA) water decreased, while the osmotically inactive (OI) water increased (Table 5.3). Blank beads compared with beads incorporating shoot-tip apices treated with all pre-treatments and a 1hr PVS2 incubation contained ca.5% less water content (FW) (Table 5.3), despite similar ice melt profiles enthalpy differences were seen (Table 5.8).

Table 5.3 shows the fresh bead weight range (21.3mg to 34.5mg). The largest difference (12.8mg) between replicates within the same treatment group (st+gly+suc-PV) was observed in beads not pre-treated with PVS2. The mean % FW water content (Table 5.3) was lowest at 71.8±0 % (blk-suc–gly-PV) and highest at 95.41±2.9% (st+gly+suc+1hr PV). The OA water content was reduced from 2.14 g/g DW to 0.67 +0.18 g/g DW following glycerol treatment to 0 g/g DW following 2hr PVS2 incubation. The OI water content increased from 0.27 g/g DW to 2.44 g/g DW following 2hr PVS2 treatment.

The reduction in OA water content following glycerol treatment showed a corresponding decrease in peak height (2/3rds to ½) and enthalpy of ice nucleation (-162.6 to -84 J/g) and their melt events (213.1 to 68.41 ±12 J/g) in Figure 5.4 a-d. PVS2 treatment for 1hr reduced ice nucleation/ melt events; PVS2 treatment for 2hr eliminated ice events and a Tg was produced on re-warming (Fig.5.4 e-h).

During cooling, untreated beads formed ice nucleation peaks at -13.2°C completing at -32.1°C; beads incorporating a shoot-tip apex, formed and ended in the lower temperature range (Table 5.8). The enthalpy was reduced from -162.6 J/g in a blank bead without any treatment to -2.016 J/g following 1hr PVS2. Glass transition (Tgs) events occurred at ca. -60°C. Upon re-warming (Table 5.9) the temperature of the onset of ice melt peaks decreased from -1.32°C (blk no treatment) to -43.9 °C (PVS2 1hr), as did the corresponding enthalpies (213.1 to 17.2 J/g). In contrast the temperature of Tg events increased from -45.52°C (no treatment) to -25.6°C (PVS2 1hr).

5.3.2 Somatic embryos/ESM

5.3.2.1 Encapsulation-dehydration

Somatic embryos/ESM were pre-treated with 0.4M sorbitol before Na-alginate encapsulation, 0.75M sucrose dehydration and laminar air-flow desiccation (0-4hr).
DSC analysis was undertaken and the data shown in three ways the size range of beads and water contents (Table 5.4), representative examples of cooling and warming profiles (Figs. 5.5 to 5.6) and summaries of the mean onset, midpoints and ends of thermal events and associated enthalpy/heat capacities for these samples (Tables 5.10 to 5.11).

Ice nucleation events were eliminated in encapsulated somatic embryos following all pre-treatments and 3hr desiccation and in encapsulated ESM following all pre-treatments and 4hr desiccation. The water content (% FW) decreased through progressive dehydration and desiccation treatments. The % RM in beads incorporating a somatic embryo following 3hr desiccation was 25% higher than blank beads in the corresponding treatment, but this water was all osmotically inactive with no detectable ice nucleation/melt events. Table 5.4 shows the bead weights (FW), which ranged from 11.3-38.3mg. The largest bead (non- dehydrated or desiccated) was re-cut to fit in the DSC pan.

The water content (Table 5.4) decreased from 77.5 $\pm$ 2.7% (blk+suc) to 26.77% (blk +suc+3hr) on a fresh weight basis. OA and OI water content patterns in encapsulated somatic embryos showed a similar response through desiccation, as did the encapsulated shoot-tip apices OA content declined from 1.88 $\pm$ 0.07 before dehydration to 0$\pm$0 g/g DW after 3hr of desiccation. OI water increased following 1hr desiccation (0.87$\pm$0.19 to 1.35$\pm$0.42 g/g DW) and then decreased slightly after 2hr desiccation (1.35$\pm$0.42 to 1.29$\pm$0.14 g/g DW). The largest bead weight difference (25mg) was observed between blank beads not desiccated in laminar air-flow, and the smallest bead weight difference (3.3mg) was observed between 3hr desiccated beads incorporating a somatic embryo (Table 5.4).

Figure 5.5 (a-d) shows a 50% reduction in nucleation/melt peak size following 1 hr desiccation, a small additional peak or step (complexity phenomena) was observed on the shoulders of the nucleation and melt peaks in Figure 5.5 (c-d). After 3hr of desiccation, these peaks were eliminated (Fig. 5.5 e-f) and other thermal steps (both exothermic and endothermic) occurred in addition to the expected Tgs. Regarding the encapsulated ESM, Tgs were the only thermal events to occur during cooling and warming especially in the 3hr desiccated, encapsulated ESM samples; no thermal instabilities were observed after 4hr

desiccation but there was a change in the baseline gradient (Fig. 5.6 a-d). Table 5.10 shows that ice nucleation occurred at -18.8 to -31.9°C and ended at -25°C to -40°C depending on bead size and treatment. Over the 2-3hr desiccation, the enthalpy of the ice nucleation peaks was reduced from 189.2 to 71 J/g. Tg events occurred at temperatures varying from -19.8 to -122.8 °C. Table 5.11 shows the temperature of the ice nucleation peak onset decreased through progressive dehydration and desiccation treatment from above 0 °C to -12.2 °C (ESM 3hr desiccation). The end of the peaks and the enthalpy values also decreased correspondingly from 17.5 to 2.6°C /192 to 62.65J/g respectively

5.3.2.2 Plant Vitrification Solution 2

Somatic embryos (sizes 2 to 3) were transferred from the donor culture to 0.4M sorbitol in solid SEIM for 48hr. The naked embryos were then directly immersed in chilled PVS2 prepared in SEIM at 0°C for 30min to 1hr. DSC analysis was undertaken and the results are shown in three ways; Table 5.5 shows the water contents, Fig. 5.7 a-d representative thermal profiles and Tables 5.12 and 5.13 summaries means of the thermal event onsets, midpoints, ends and associated enthalpies of the samples. No ice nucleation/melt events were observed following 30min to 1hr PVS2 incubation (Table 5.12). The water content (FW) of somatic embryos was halved from 32.5% to 16.67 % with 30min PVS2 application (Table 5.5) resulting in the glass transition events seen in Fig 5.7. Additional thermal steps (inverted thermal steps circled, relative to Tg events) were observed in all profiles, between -50 and -75°C (Fig. 5.7a-d) indicating the complexity of PVS2 thermal characteristics.

Table 5.5 show the fresh weight of naked embryos (mg), which ranged from 1.2 mg to 5mg. The largest embryos were in the 1hr PVS2 treatment group, and overall the % RM decreased from 32.5 to 10%. The OA water without PVS2 incubation was 0.3 g/g DW and was reduced to 0 g/g DW following PVS2 incubation (Table 5.5). The OI water content also decreased from 0.5 g/g DW to 0.11 g/g DW following 1hr PVS2 incubation. Ice was formed in cooling (-16 to -17°C) and re-warming (-0.6 to 8°C) in all embryos pre-treated with sorbitol only (Fig. 5.7a-b, Tables 5.12 and 5.13). Tgs occurred during cooling at around -104 to -130°C and during warming at -50°C. A destabilisation event occurred in the 30min PVS2 treated embryo at 0°C (enthalpy 26.14 J/g) (Table 5.12 and 5.13).

5.3.2 Summary Points

- Differences in sample sizes resulted in different thermal profiles.

- Water content and ice formation were reduced through increasing dehydration and desiccation.
- Cold hardening, sorbitol pre-treatment, sucrose incubation, DMSO and PVS2 treatment reduced the total water and influenced the osmotically active water content.
- There were differences in the OI water content following laminar air-flow desiccation (Table 5.14).
- Ice was not formed in any explants desiccated for 4hr.
- Tgs and ice profiles can occur in the same bead.
- Other thermal events occurred, some were de-vitrification peaks during heating.
- Thermal stability correlated well with successful protocols. Optimised encapsulation-dehydration protocols for shoot-tip apices (Chapter 4) incorporated sucrose, cold hardening and DMSO pre-culture with 4hr laminar desiccation, showed no OA water and stable glass transitions. Optimised encapsulation-dehydration and PVS2–vitrification protocols, for somatic embryos (Chapter 3) incorporated a sucrose pre-treatment and 3–4hr laminar air-flow desiccation or osmoprotection in a loading solution followed by PVS2 vitrification for 30 min both showed no OA water and stable glass transitions.

Table 5.2 Water content of Na–alginate encapsulated shoot-tip apices from *Picea sitchensis* following cold-hardening, sucrose dehydration, DMSO treatments and laminar air-flow desiccation

Treatment	Lam. air desic. (hr)	Bead wt*range (mg)	Relative moisture (% RM)	Water Content (g/g DW)	OA Water Content[1] (g/g DW)**	OI Water Content[2] (g/g DW)**
Blk –*suc*	0	25.7-35.9	72.12± 3.16	3.04±0.28	2.12±0.11	0.91±0.2
Blk+suc	0	25.2-27.3	57.55±3.24	1.38±0.19	0.857+0.03	0.53+0.2
st-*suc*+ch+DM	0	14.1-26.5	61.01±5.43	1.67±0.41	1.28±0.3	0.39±1.3
st+suc+ch+DM	0	35.7-40	56.8±9.28	1.57±0.6	0.84±0.31	0.73±0.3
Blk-*suc* *	3	12.6-15	59.57±2.42	1.48±0.14	0.29±0.29	1.18±0.5
Blk +suc	3	27.7-29.2	55.42±6.11	1.32±0.28	0.001±0.0	1.32±0.3
st-*suc*+ch+DM	3	6.5-19	63.05±4.48	1.79±0.36	0.709±0.70	1.09±0.4
st+suc+ch-*DM*	3	25.9-29	31.03±1.03	0.45±0.02	0±0	0.45±0.02
st+suc-ch+DM	3	27.6-27.7	63.85±0.7	1.08±0.012	0±0	1.08±0.01
st+suc+ch+DM	3	15.8-24	52.76±6.76	1.044±0.23	0±0	1.04±0.23
st+suc+ch+DM	4	20.6-22	42.19±2.64	0.89±0.05	0±0	0.893±0.05

blk= Blank bead; st=Shoot-tip apex; suc =0.75M sucrose incubation for 18hr; ch =cold hardening 1 week culture in alternating 8hr light + 20°C and 16hr dark+ 4°C; DM= pre-treatment 5% (v/v) DMSO 12hr.

* Beads that had not been desiccated or had only been laminar-air desiccated for 1-2hr were cut into sections to fit the DSC pan; the explant or a section of the explant was included in this bead section. Beads that had been laminar-air desiccated for 2hr or more were not sectioned, as they were a sufficiently small to fit the DSC pan.

** OA= Osmotically Active water; OI=Osmotically Inactive water

1 Calculated by dividing the melt endotherm peak area (J) by 334.5 J/g; 1g of water releases/absorbs 334.5J of energy during the transition between liquid and ice (Block, 2003)

2 Calculated as the difference between total and osmotically inactive water contents

Means ± SEM are derived from 3 replicate (beads) *= 2 replicate (beads)

Italic = without critical component

Table 5.3 Water content of Na–alginate encapsulated shoot-tip apices *Picea sitchensis* following sucrose and PVS2 exposure.

Treatment	**PVS2 (hr)**	**Bead wt range (mg)**	**Relative Moisture (%RM)**	**Water Content (g/g DW)**	**OA Water Content[1] (g/gDW)***	**OI Water Content (g/g DW)***
blk-suc-PV	0	28	71.78+0	2.42+0	2.14+0	0.27+0
st+gly+suc-PV	0	21.7-34.5	82.3+0.7	2.68+0.38	0.67+0.18	2.01+0.27
blk +gly+suc+PV	1	26-29	90.709+0.32	3.00+0.17	0.12+0.04	2.08+0.12
st+gly+suc+PV	1	21.3-24.1	95.41+2.9	2.85+0	0.15+0	2.7+0
st+gly+suc+PV	2	25.1	80.87+0	2.4+0	0	2.44+0

st=Shoot-tip apex; suc =0.75M sucrose incubation for 18hr; PV= PVS2 solution; gly= 2M glycerol for 30min.

* As for Table 5.2

Table 5.4 Water content of Na–alginate encapsulated *P. sitchensis* somatic embryos/ESM following sucrose dehydration and laminar air-flow desiccation.

Treatment	Lam. air desic. (hr)	Bead wt range (mg)	Relative Moisture (%RM)	Water Content (g/g DW)	Osmotically Active Water Content[1] (g/g DW)*	Osmotically Inactive Water Content[2] (g/g DW)*
blk +suc	0	13.9-38.3	77.5±2.7	2.95±0.37	2.26±0.23	0.69±0.15
se +suc	0	17.8-35	59.9±12.6	2.75±0.27	1.88±0.07	0.87±0.19
se+suc	1	23-38	53.38±5.56	2.01±0.686	0.65±0.26	1.35±0.42
se+suc	2	19-25	52.7±5.58	1.35±0.16	0.06±0.06	1.29±0.14
Blk+suc	3	14.2-23	26.77±7.26	0.70±1.6	0.16±0.16	0.54±0.03
se +suc	3	20.6-24	50.54±6.29	1.08±0.036	0±0	1.1±0.04
ESM+suc	3	11.3-16.8	37.38±10.8	0.66±0.307	0.18±0.18	0.4±0.12
ESM+suc	4	11.3	40.707±0	0.554±0	0±0	0.5±0

blk= Blank Na-alginate bead prepared with SEMM (embryogenic suspensor masses)/SEIM (Somatic embryos) media

se=Somatic embryo; suc =0.75M sucrose incubation for 18hr, on a rotary shaker at 20°C, prepared with SEMM/SEIM media

ESM =Embryogenic suspensor masses

* As for Table 5.2

Table 5.5 Water content of somatic embryos of *Picea sitchensis* following sucrose dehydration and PVS2 exposure.

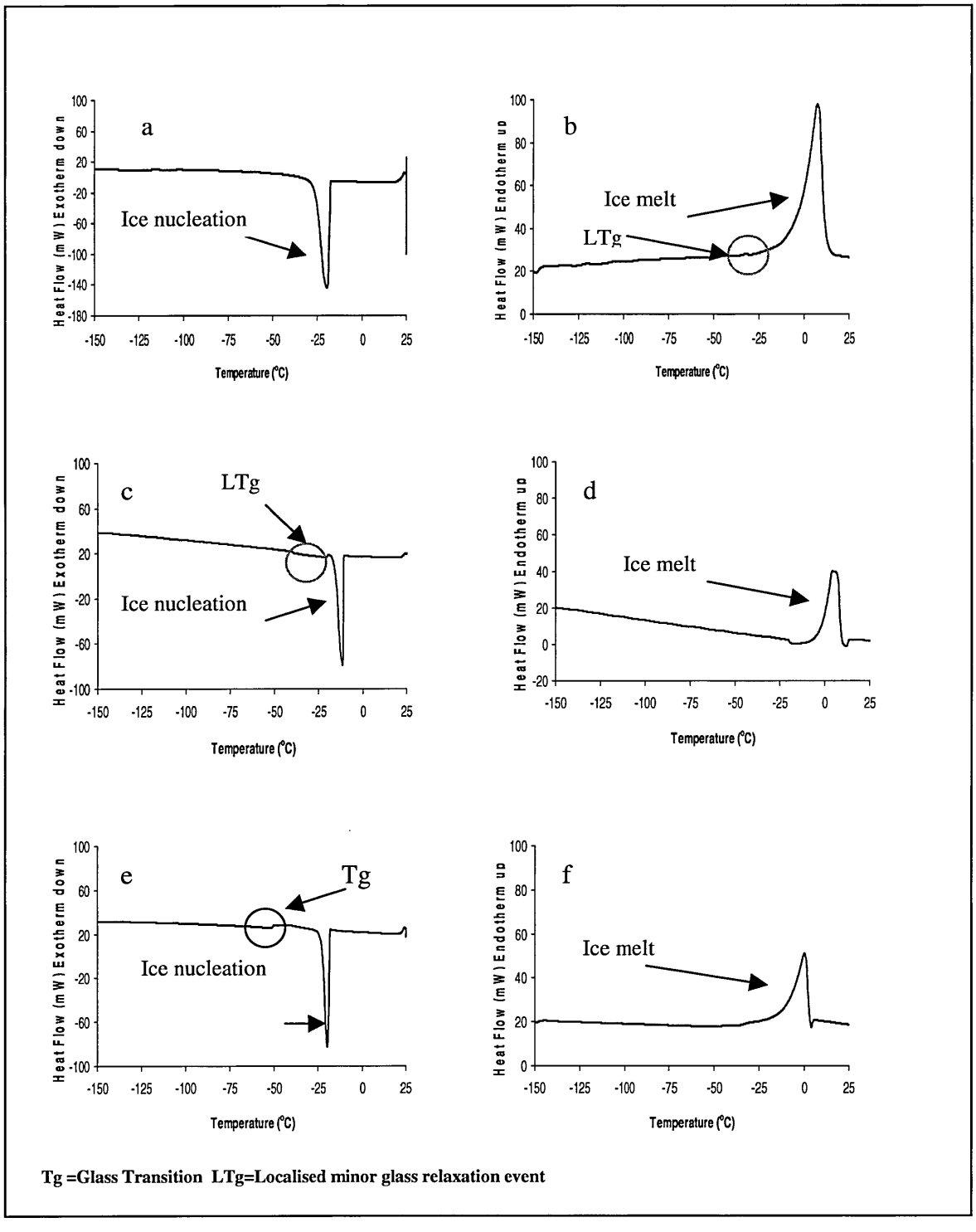
Treatment	**PVS2 (hr)**	**Embryo wt range**	**Relative Moisture (%RM)**	**Water Content (g/g DW)**	**Osmotically Active Water Content[1] (g/g DW)***	**Osmotically Inactive Water Content[2] (g/g DW)***
Se-PV	0	1.6-4	32.5±32.5	0.812±0.812	0.3±1.6	0.5±0.64
Se+PV	0.5	1.2	16.67±0	0.2±0	0±0	0.2±0
Se+PV	1	5	10±2	0.11±0.2	0±0	0.11±0.02

Se=Somatic embryo; suc =0.75M sucrose incubation for 18hr; Means ± SEM are derived from 3 replicate (beads) *= 2 replicate (beads), PV= PVS2 solution 30min

Na-alginate beads in SEIM media,

* As for Table 5.2

Figure 5.2 DSC thermograms of blank Na alginate beads and encapsulated shoot-tip apices (without laminar air-flow desiccation).

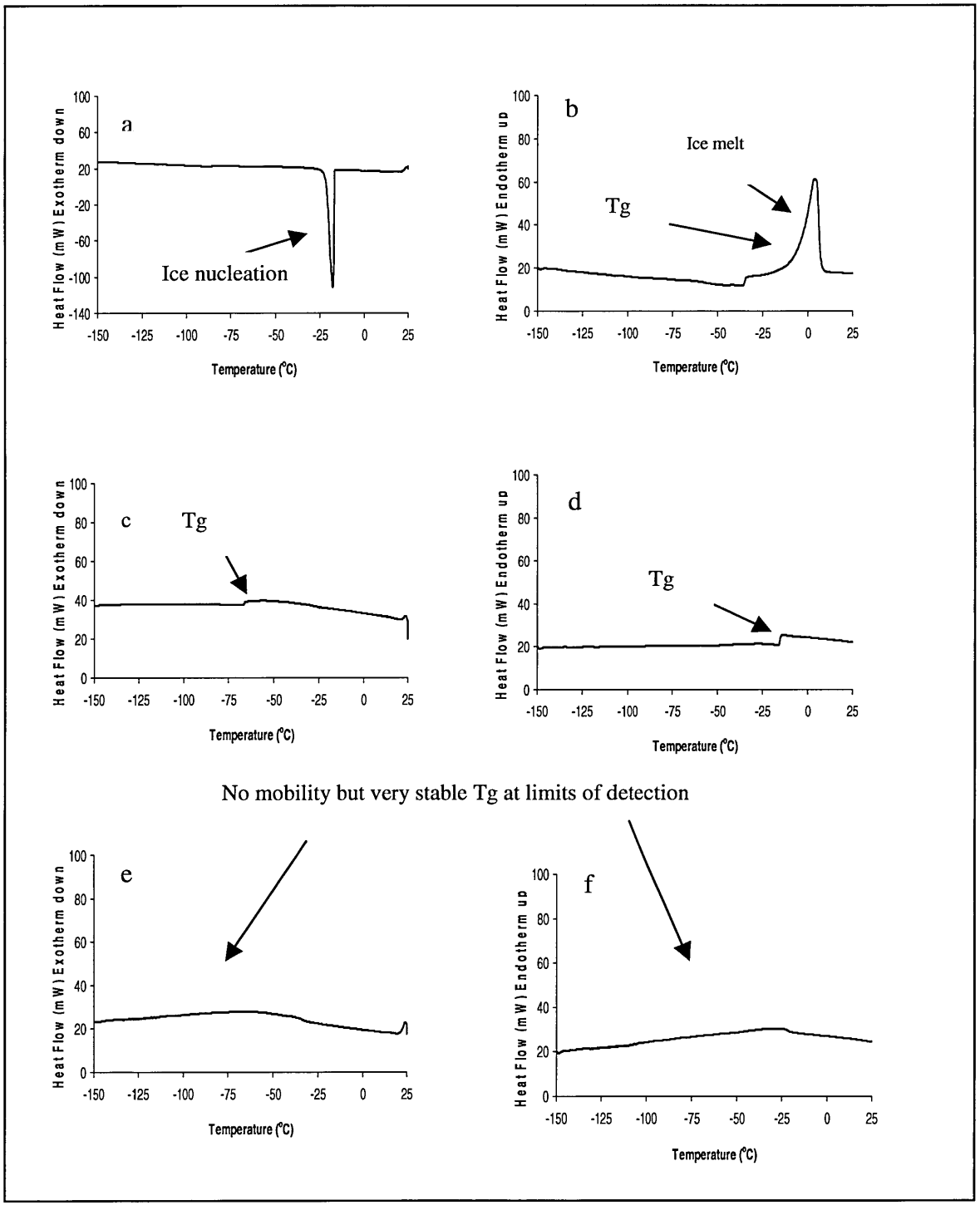


Tg =Glass Transition LTg=Localised minor glass relaxation event

Cooling profile (-10°C/min) from 25°C to -150°C, heating profile (+10°C/min) from-150°C to 25°C.

a) Blank bead no sucrose no desiccation cool b) heat profile of *a* c) shoot-tip apex in bead no sucrose no desiccation cool d) heat profile of *c* e) shoot- tip apex in bead + 0.75M sucrose 18hr osmotic dehydration no desiccation cool f) heat profile of *e*

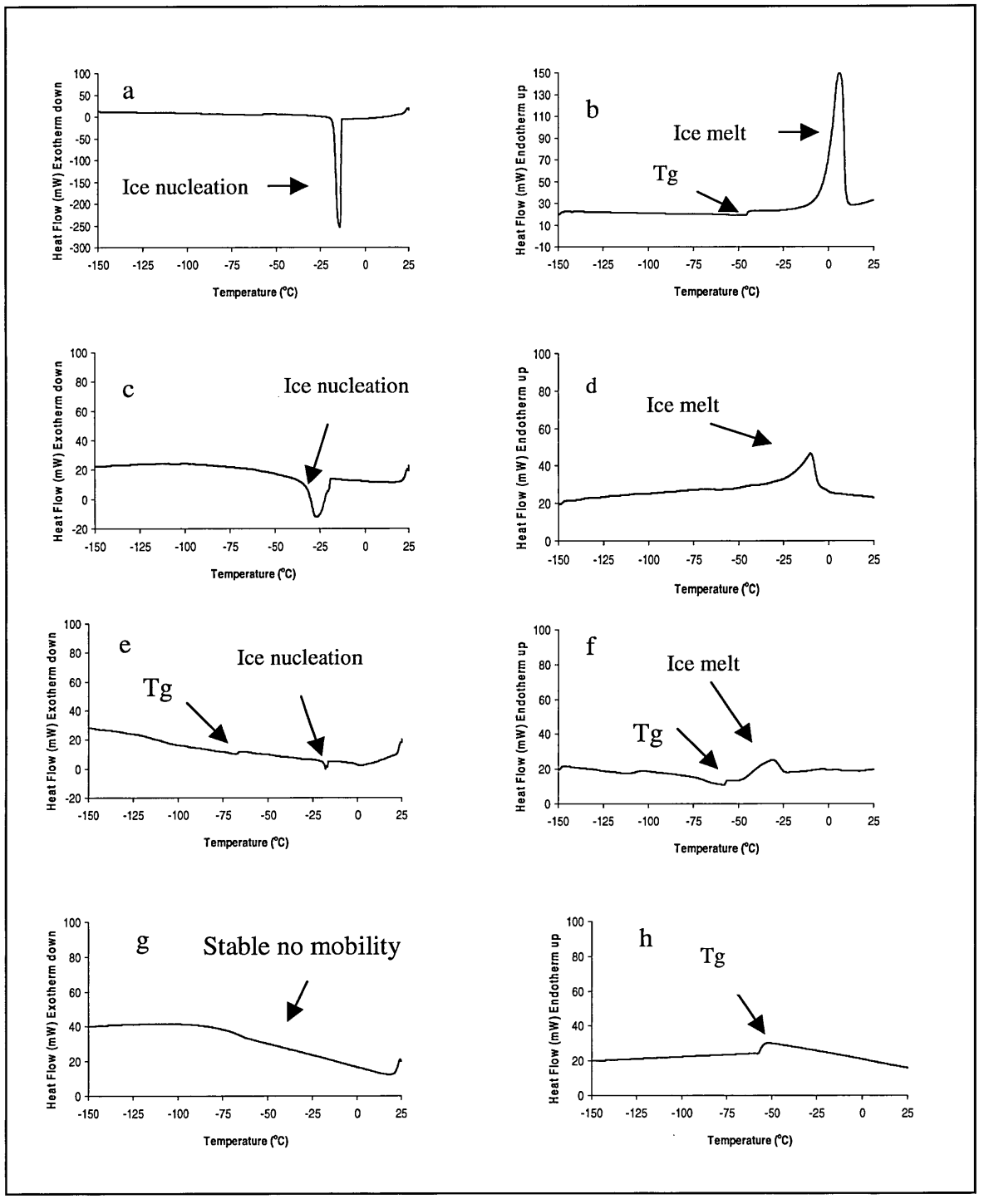
Figure 5.3 DSC thermograms of Na–alginate encapsulated shoot-tip apices (with laminar air-desiccation)



Cooling profile (-10°C/min) from 25°C to -150°C, heating profile (+10°C/min) from-150°C to 25°C.

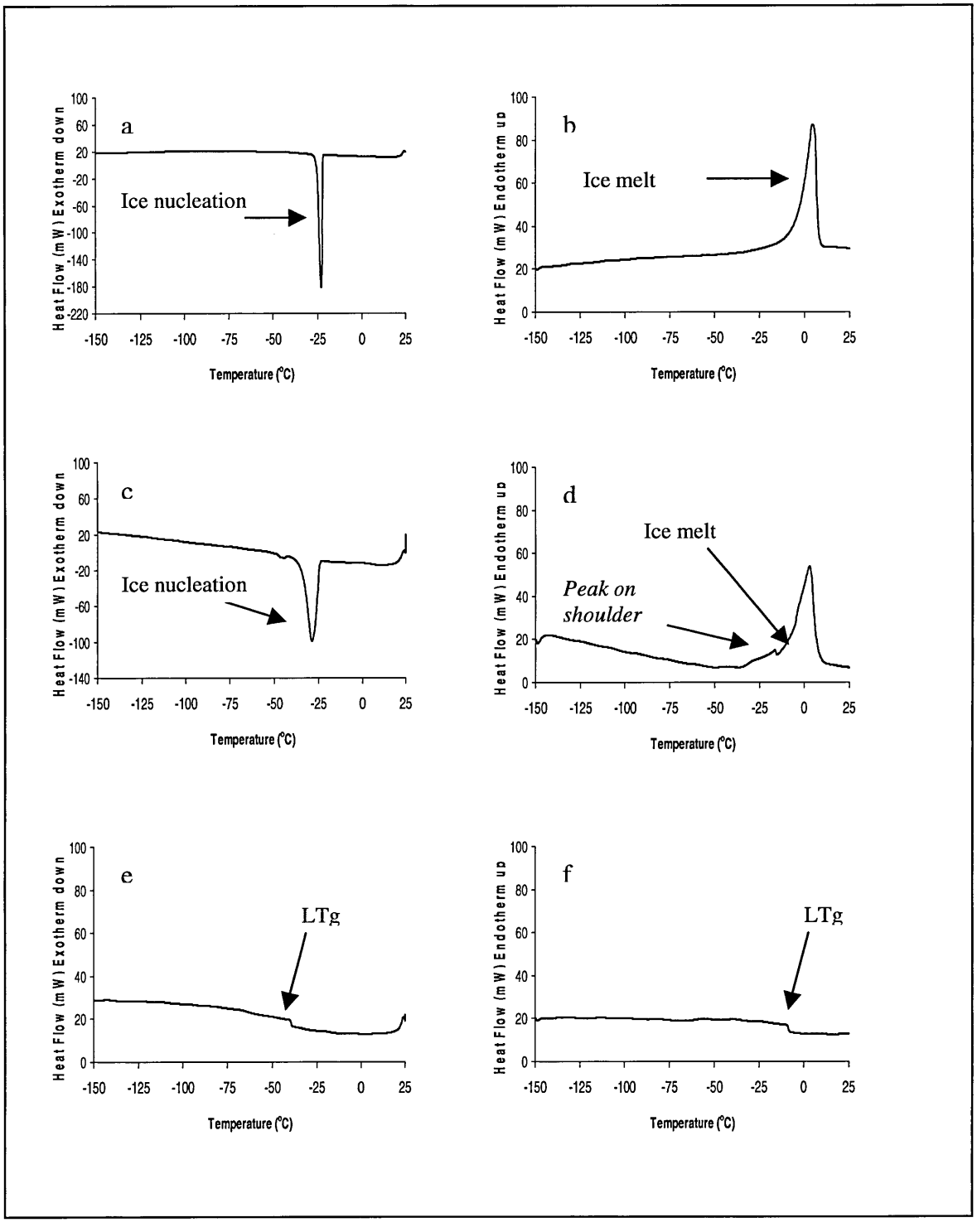
(a) Shoot-tip apex encapsulated in Na-alginate beads with no sucrose dehydration and 3hr desiccation cool (b) heat profile of *a* (c) shoot-tip apex in bead after 0.75M sucrose dehydration +3hr desiccation treatments cool (d) heat profile of *c* (e) shoot-tip apex in bead + 0.75M sucrose dehydration, 18hr osmotic dehydration +4hr desiccation cool (f) heat profile of *e*

Figure 5.4 DSC thermograms of Na–alginate encapsulated shoot-tip apices (encapsulation-vitrification)



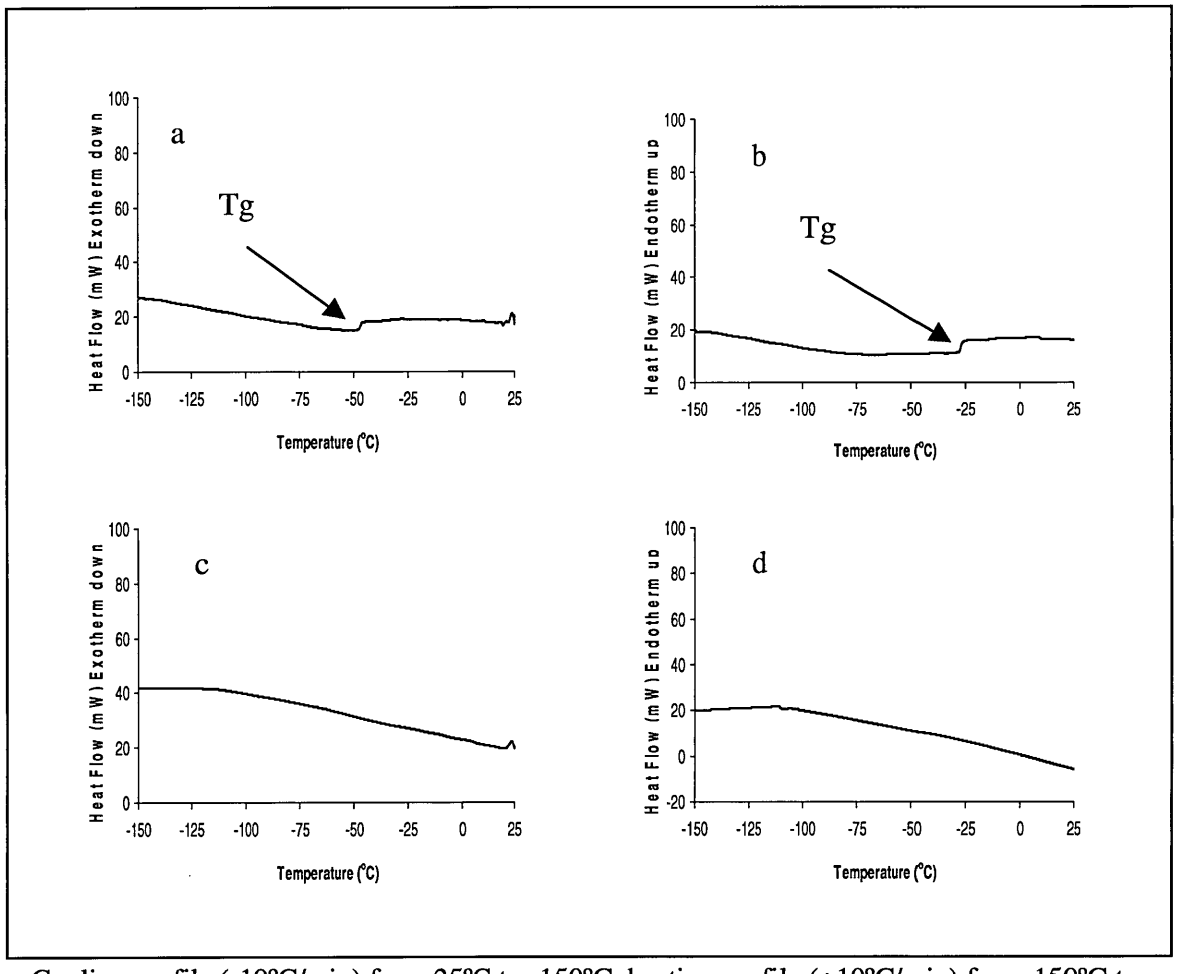
Cooling profile (-10°C/min) from 25°C to -150°C, heating profile (+10°C/min) from-150°C to 25°C.
(a) Shoot-tip apex encapsulated in Na-alginate with 0.4M sucrose, 1 week cold hardening, 5%(v/v) DMSO 12hr (b) heat profile of *a* (c) Shoot-tip apex encapsulated in Na-alginate with 0.4M sucrose, 1 week cold hardening, 5%(v/v) DMSO, 0.4M sucrose +2M glycerol 0°C 2hr. (d) heat profile of *d* (e) Shoot-tip apex encapsulated in Na-alginate with 0.4M sucrose, 1 week cold hardening, 5%(v/v) DMSO, 0.4M sucrose +2M glycerol 0°C 2hr, PVS2 1hr (f) heat profile of *e* (g) Shoot-tip apex encapsulated in Na-alginate with 0.4M sucrose, 1 week cold hardening, 5%(v/v) DMSO, 0.4M sucrose +2M glycerol 0°C 2hr, PVS2 2hr (h) heat profile of *g*

Figure 5.5 DSC thermograms of Na–alginate encapsulated somatic embryos (encapsulation-dehydration)



Cooling profile (-10°C/min) from 25°C to -150°C, heating profile (+10°C/min) from-150°C to 25°C
(a) Na-alginate encapsulated somatic embryo no laminar air desiccation cool (b) heat profile of *a* (c) Na-alginate encapsulated somatic embryo 1hr desiccation cool (d) heat profile of *d* (e) Na-alginate encapsulated somatic embryo 3hr desiccation cool, (f) heat profile of *e*
LTg= Localised minor glass relaxation event

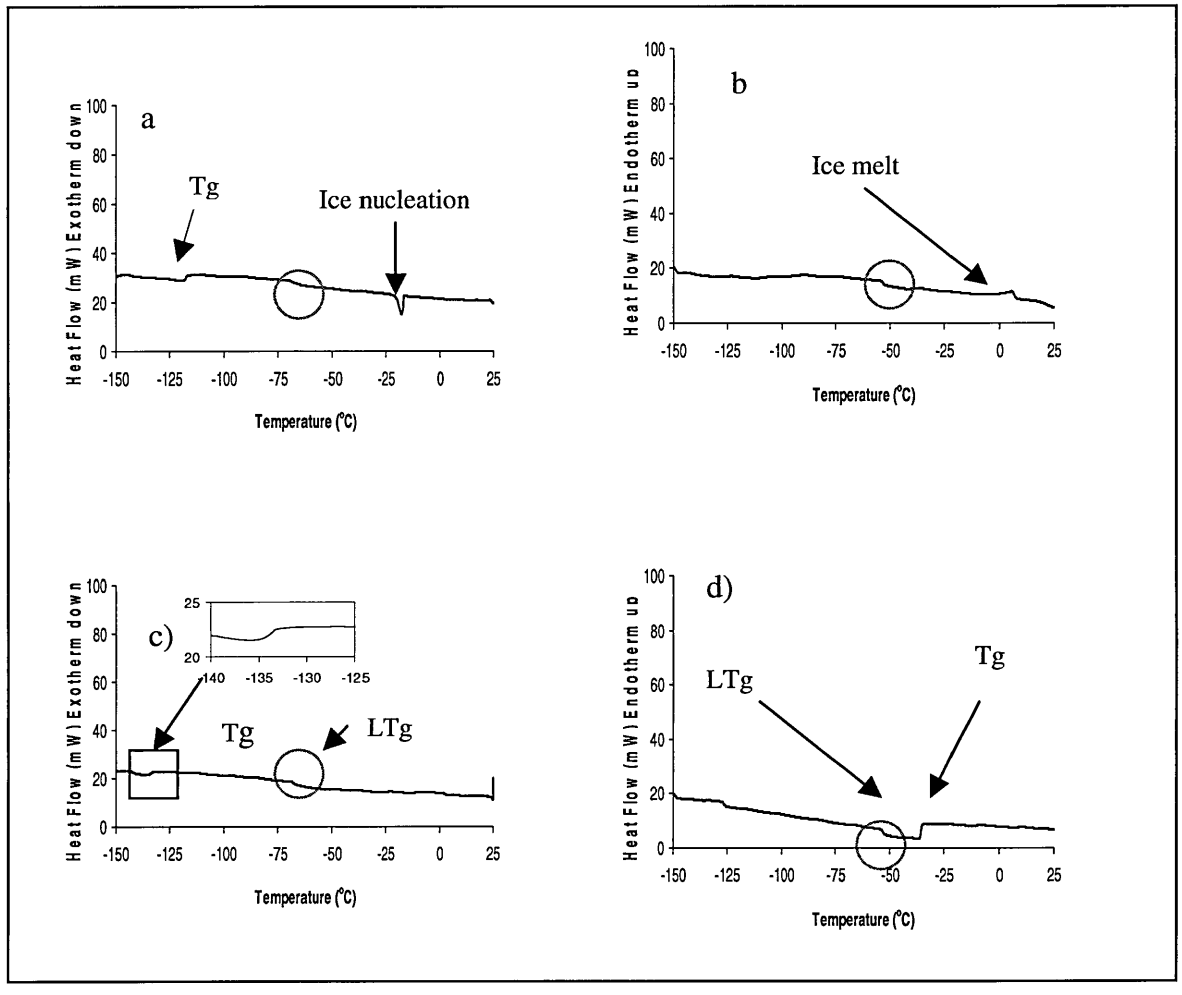
Figure 5.6 DSC thermograms Na–alginate encapsulated Embryogenic suspensor masses (ESM) Encapsulation-dehydration



Cooling profile (-10°C/min) from 25°C to -150°C, heating profile (+10°C/min) from-150°C to 25°C.

(a) Na-alginate encapsulated embryogenic suspensor masses (ESM) 0.4M sorbitol pre-treatment 48hr in SEM, 0.75M sucrose in SEM 18hr and 3hr laminar air-flow desiccation cool (b) heat profile of *a* (c) Na-alginate encapsulated ESM all treatments 4hr desiccation cool (d) heat profile of *c*

Figure 5.7 DSC thermograms of non-encapsulated somatic embryos following encapsulation–vitrification (PVS2)



Cooling profile (-10°C/min) from 25°C to -150°C, heating profile (+10°C/min) from-150°C to 25°C.

a) Somatic embryo no PVS2 cool b) heat profile of *a* c) somatic embryo 1hr PVS2 cool d) heat profile of *c*

Tg= Glass Transition; LTg= Localised minor glass relaxation event. Circles indicate LTg

Table 5.6 Thermodynamic cooling properties of shoot-tip apices from *P. sitchensis* (encapsulation-dehydration)

Treatment	Desic. (hr)	Thermal Event (Reps)	Ice Nucleation –Peak				Glass Transition- (TG)			
			Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
blk-suc (2 rps)	0	ICE(2/2) TG(1/2)	-13.3±1.8	-19.1±1.3	-24.9±0.8	-255.7±5.3	-85.106±0	-85.68±0	-86.26±0	0.359±0
blk+suc	0	ICE(3/3) TG(1/3)	-16.8±0.7	-21.4±0.4	-25.9±0.5	-145.4±4.4	-63.0±0	-63.72±0	-64.43±0	0.375±0
st-suc	0	ICE(3/3) TG(3/3)	-12.2±0.9	-14.8±1.7	18.4±1.9	-169.4±26	-53.5±17	-53.9±16.9	-54.4±16.9	0.91±0.4
st+suc	0	ICE (3/3) TG(3/3)	-16.8±0.8	-18.8±0.5	-22.9±1.1	-117.5±28	-50.13±0	-50.6±0	-51.1±0	0.52±0
blk-suc	3	ICE(3/3) TG(2/3)	-18.2±1.4	-20.9±1.4	-23.6±1.3	-121.7±24.5	-89.5±1.3	-90.2±1.2	-90.9±1.1	0.8±0.3
blk+suc (2 rps)	3	TG(2/3)	-	-	-	-	-103.1±18.2	-85.1±18.3	-85.7±18.3	0.4±0.13
st-suc+ch+DM	3	ICE(3/3) TG(2/3)	-15.8±0.4	-18.6±0.2	-21.5±0.2	152.3±43.4	-108.5±8.2	-109.0±8.3	-109.6±8.3	1.01±0.3
st+suc+ch-DM	3	TG(1/3)	-	-	-	-	-122.7+0	-112.3±0	-111.7±0	0.53±0
st+suc+DM-ch	3	TG(3/3)	-	-	-	-	-88.7±4.9	-89.3±5.01	-89.8±5.01	0.43±0.06
st+suc+DM+ch	3	TG(3/3)	-	-	-	-	-83.7±20.6	-84.6±20.6	-84.8±20.6	0.48±0.1
st+suc+DM+ch	4	TG 2/3)	-	-	-	-	-49.7±35.6	-50.2±35.6	-50.7±35.6	0.4±0.13

ICE= Ice nucleation; TG = Glass transition; st= shoot-tip apex; blk= blank Na-alginate bead prepared in MS media; suc =0.75M sucrose incubation for 18hr at 20°; c=cold hardening 1 week light/dark alternation; DM= 5% (v/v) DMSO; Reps = Number of replicates showing corresponding thermal event

Desic=Laminar air desiccation hr

Treatment includes: ± cold hardening, 5% (v/v) DMSO and encapsulation with alginate ± 0.75M sucrose 18hr dehydration and air-flow desiccation (0-4hr). Means ± SEM derived from 2-3 beads

Table 5.7 Thermodynamic heating properties of shoot-tip apices from *P. sitchensis* (encapsulation-dehydration)

Treatment	Desic. (hr)	Thermal Event (Reps)	Ice Nucleation –Peak				Glass Transition- (TG)			
			Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
blk-suc (2 replicates)	0	ICE(2/2) TG(1/2)	4.8±0.06	13.2±0.0	21.5±0.0	140.5±36.4	-19.7±0	-19.7±0	-18.6±0	0.61±0
blk+suc	0	ICE(3/3) TG(2/3)	-0.6±0.6	6.3±0.9	13.2±1.2	107.8±1.2	-32.3±0	-31.8±0	-31.3±0	0.49±0
st-suc	0	ICE(3/3) TG(1/3)	-0.1±1.07	5.9±1.7	12.0±2.3	154.08±22.9	-43.7±5.2	-43.2±5.2	-42.6±5.09	0.72±0.20
st+suc	0	IC (3/3)	-3.5±1.9	2.0±2.2	7.6±2.5	81.7±2.3	-	-	-	-
blk-suc	3	ICE(3/3) TG(2/3)	-7.5±3.2	-1.2±2.7	5.0±2.2	87.9±12.4	-57.1±0.32	-56.6±0.34	-55.9±0.35	1.4±0.1
blk+suc (2 replicates)	3	ICE(2/2)	-22.9±2.8	-17.0±0.7	11.0±1.4	10.02±8.9	-32.7±0.8	-31.2±0.9	-29.7±0.9	0.5±0.1
st-suc+ch+DM	3	ICE(3/3) TG(3/3)	-3.5±2.0	2.5±2.3	8.6±2.5	12.9±41.0	-25.5±6.4	-25.03±6.4	-24.5±6.4	2.0±0.5
st+suc+ch-DM	3	TG(3/3)	-	-	-	-	-32.9±12.0	-31.6±11.8	-30.3±11.7	1.5±0.2
st+suc+DM-ch	3	TG(3/3)	-	-	-	-	-45.1±6.5	-44.6±6.4	-44.1±6.4	1.0±0.1
st+suc+DM+ch	3	TG(3/3)	-	-	-	-	-31.2±12.0	-31.1±12.0	-31.0±12.0	1.2±0.5
st+suc+DM+ch	4	TG(1/3)	-	-	-	-	-58.5±0	-57.9±0	-57.4±0	0.6±0

Footnote:
ICE= Ice nucleation; TG = Glass transition st= shoot-tip apex; blk= blank Na-alginate bead prepared in MS media; suc =0.75M sucrose incubation for 18hs at 20°; ch=cold hardening 1 week light/dark alternation; DM= 5% (v/v) DMSO; Reps = number of replicates showing corresponding thermal event
Desic=Laminar air desiccation hr
Treatment includes: ± cold hardening, 5% (v/v) DMSO and encapsulation with alginate ± 0.75M sucrose 18hr dehydration and air-flow desiccation (0-4hr). Means ± SEM derived from 2-3 beads.

Table 5.8 Thermodynamic cooling properties of shoot-tip apices from *P. sitchensis* (encapsulation-vitrification-PVS2)

Treatment	Time exposure (hr)		Thermal Event (Reps)	Ice Nucleation –Peak				Glass Transition- (TG)			
	Ld Soln	PV		Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
blk-gly-PV	0	0	ICE(1/1) TG(1/1)	-13.2±0	-14.2±0	-17.4±0	-162.6±0	-57.4±0	-57.7±0	-60.0	0.661
st+gly-PV	2	0	ICE(3/3) TG(2/3)	-22.9±2.1	-26.9±0.8	-32.14±0.4	- 84.15±15.6	- 63.91±15.6	- 64.5±15.6	-64.9±15.6	0.6±0.3
blk+gly+PV	2	1	TG(2/2)	-	-	-	-	-71.8±21.4	- 72.3±21.4	-72.8±21.4	0.6±0.2
st+gly+PV	2	1	ICE(1/2)	-16.31±0	-18±0	-19.06±0	-2.0±0	-66.28±0	-66.7±0	-67.32±0	0.63±0
st+gly+PV	2	2		-	-	-	-	-	-	-	-

Footnote:
ICE= Ice nucleation; TG = Glass transition; st= shoot-tip apex; blk= blank Na-alginate bead prepared in MS media +0.4M sucrose; gly= 2hr incubation in 2M glycerol +0.8M sucrose at 0°C; PV= Plant Vitrification Solution 2 (0-2hr) at 0°C; Reps = Number of replicates showing corresponding thermal event Ld soln =time (in hr) loading solution incubation in 2M glycerol +0.8M sucrose at 0°C

Treatment included: cold hardening, 5% (v/v) DMSO and encapsulation with alginate incorporating 0.4M sucrose, ± 2M glycerol + 0.8M sucrose 2hr incubation at 0°C and ± PVS2 (0-2hr). Means ± SEM derived from 2-3 beads

Table 5.9 Thermodynamic heating properties of *P. sitchensis* shoot-tip apices (encapsulation-vitrification-PVS2)

Treatment	Time exposure (hr)		Thermal Event (Reps)	Ice Melt –Peaks				Glass Transition- (TG)			
	Ld Soln	PV		Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
blk-gly-PV	0	0	ICE(1/1) TG(1/1)	-1.32±0	6.4±0	9.73±0	213.2±0	-45.52	-45.1±0	-44.7±0	0.52
st+gly-PV	2	0	ICE(3/3) TG(1/3)	- 17.6±2.2	-5.8±2.3	-2.1±2.4	68.4±12.2	-27.41	-27.13	-26.94	0.34
blk+gly+PV	2	1	ICE(2/2)	- 34.5±1.8	- 24.7±0.1	- 21.6±1.12	12.2±4.07	-	-	-	-
st+gly+PV	2	1	ICE(1/2) TG(1/2)	-43.9±0	-31.3±0	-25.2±0	17.2±0	-25.5±0	-24.1±0	-22.9±0	1.67
st+gly+PV	2	2	TG (1/2)	-	-	-	-	-55.3 ± 0	-52.0±0	-50.0 ±0	2.3

ICE= Ice nucleation; TG = glass transition; st= shoot-tip apex; blk= blank Na-alginate bead prepared in MS media +0.4M sucrose; gly= 2hr incubation in 2M glycerol +0.8M sucrose at 0°C; PV= Plant Vitrification Solution 2 (0-2hr) at 0°C; Reps = number of replicates showing corresponding thermal event; Ld soln =time in (hr) loading solution incubation in 2M glycerol +0.8M sucrose at 0°C

Treatment included: cold hardening, 5% (v/v) DMSO and encapsulation with alginate incorporating 0.4M sucrose, ± 2M glycerol + 0.8M sucrose 2hr incubation at 0°C and ± PVS2 -2hr. Means ± SEM derived from 2-3 beads

Table 5.10 Thermodynamic cooling properties of somatic embryos from *P. sitchensis* (encapsulation-dehydration)

Treatment	Desic. (hr)	Thermal Event (Reps)	Ice Nucleation –Peak				Glass Transition- (TG)			
			Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
blk +suc	0	ICE(3/3) TG(1/3)	-17.9±2.0	-20.5±2.1	-25.8±2.3	189.2± 6.6	-77.1±0	-78.9±0	-80.8±0	0.21±0
se+suc	0	ICE(3/3)	-18.8±1.7	-20.2±1.5	-24.9±0.5	164.9±15.0	-	-	-	-
se+suc	1	ICE(2/2) TG(1/2)	-31.9+7.6	-36.1±7.6	-41.51±7.8	118.8±36.05	-42.9±0	-43.4±0	-43.7±0	0.35±0
se+suc	2	ICE(1/3) TG(1/3)	-22.19±0	-25.17±0	-28.15±0	71.9±0	-19.34	-19.81	-20.28	0.72±0
se+suc	3	TG(1/3)	-	-	-	-	-122.2±0	-122.8±0	-123.5±0	0.82±0
blk+suc	3	ICE(1/3) TG(1/3)	-24.5±0	-26.36±0	-29.20±0	76.59±0	-69.7±0	-70.34±0	-70.8±0	0.51±0
ESM +suc	3	ICE(1/2) TG(2/2)	-25.7±0	-27.7±0	-32.3±0	85.4±0	-72.6±26.4	-73.2±26.4	-73.9±26.5	0.95±0.3
ESM +suc	4	ICE(1/1)	-	-	-	-	-	-	-	-

Footnote:
ICE= Ice nucleation; TG = glass transition; se= somatic embryo; ESM =embryogenic suspensor mass; blk= blank Na-alginate bead prepared in SEIM media; Suc =0.75M sucrose incubation for 18hr at 20° C; Reps = Number of replicates showing corresponding thermal event
Desic=Laminar air desiccation hr
Treatment includes: encapsulation with alginate ± 0.75M sucrose 18hr dehydration and air-flow desiccation (0-4hr). Means ± SEM derived from 2-3 beads

Table 5.11 Thermodynamic heating properties of somatic embryos from *P. sitchensis* (encapsulation-dehydration)

Treatment	Desic. (hr)	Thermal Event (Reps)	Ice Nucleation –Peak				Glass Transition- (TG)			
			Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
blk +suc	0	ICE(3/3) TG(2/3)	1.21±0.9	13.7±1.7	17.52±1.3	-192.22±7.8	-60.32±6.1	-59.6±6.1	-58.2±6.6	0.6±0.03
se+suc	0	ICE(3/3)	-0.9±1.5	9.02±2.4	12.3±2.5	-157.8±13.8	-	-	-	-
se+suc	1	ICE(2/2) TG(2/2)	-13.7±6.6	-2.13±5.2	2.43±4.7	41.4±25.6	-26.78±2.1	-17.55±3.4	-8.3±8.9	0.19±0
se+suc	2	ICE(2/2) TG(2/2)	-18.9±8.4	-10.7±6.3	-2.47±4.2	35.4±15.8	-46.62±0	-46.09±0	-45.6±0	1.26±0
se+suc	3	TG(2/3)	-	-	-	-	-40.5±6.5	-40.0±6.5	-39.48±6.5	1.93±0.1
blk+suc	3	ICE(1/3) TG(1/3)	-10.8±0	-0.8±0	2.25±0	66.1±0	-43.184+0	-42.64±0	-42.05±0	0.74±0
ESM+suc	3	ICE(1/2)	-12.2	-0.8	2.69	62.65	-50.3±22.6	-49.3±22.2	-48.6±22.3	1.5±0.6
ESM+suc	4	TG(1/1)	-	-	-	-	-108.3±0	-107.4±0	-106.3±0	0.35±0

Footnote:
ICE= Ice nucleation; TG = Glass transition; se= somatic embryo; ESM =embryogenic suspensor mass; blk= blank Na-alginate bead prepared in SEIM media; suc =0.75M sucrose incubation for 18hr at 20° C; Reps = Number of replicates showing corresponding thermal event
Desic=Laminar air desiccation hr

Treatment includes: encapsulation with alginate ± 0.75M sucrose 18hr dehydration and air-flow desiccation (0-4hr). Means ± SEM derived from 2-3 beads

Table 5.12 Thermodynamic cooling properties of *P. sitchensis* somatic embryos (non-encapsulated PVS2 vitrified)

Treatment	PVS2 (hr)	Thermal Event (Reps)	Ice Nucleation –Peak				Glass Transition- (TG)			
			Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
se- PV		ICE(3/3) TG(2/3)	- 16.9±1.3	- 16.8±0.3	- 17.9±0.8	65.4±21.8	-104.4±12.5	-104.9±12.5	-105.5±12.6	5.25±2.0
se + PV	0.5	(1/1)	-	-	-	-	-	-	-	-
se + PV	1	TG(2/2)	-	-	-	-	-129.9±3.1	-130.5±3.3	-131.2±3.5	1.9±0.5

ICE= Ice nucleation; TG = Glass transition; se= somatic embryo; PV= Plant Vitrification Solution 2 (0-2hr) at 0°C; Reps = number of replicates showing corresponding thermal event

Treatments included: all somatic embryos subject to loading solution (2M glycerol + 0.4M sucrose 1hr incubation at 0°C) ± PVS2 (0-2hr). Means ± SEM derived from 2-3 somatic embryos

Table 5.13 Thermodynamic heating properties of *P. sitchensis* somatic embryos (non-encapsulated PVS2 vitrified)

Treatment	PVS2 (hr)	Thermal Event (Reps)	Ice Nucleation –Peak				Glass Transition- (TG)			
			Onset (°C)	Midpoint (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	Midpoint (°C)	End (°C)	Heat Capacity (J/g*°C)
se- PV	None	ICE(3/3) TG(2/3)	-0.6±0.19	4.95±1.7	8.16±0.14	167.21±83.0	-50.9±10.2	-50.5±10.2	-50.0±10.2	1.742
se + PV	0.5	TG(1/1)	-	-	-	-	-0.394	0.15	0.551	26.14
se + PV	1	TG(2/2)	-	-	-	-	-31.37±4.7	-30.84±4.7	-30.39±4.7	6.6±0.03

ICE= Ice nucleation; TG = Glass transition; se=somatic embryo; PV= Plant Vitrification Solution 2 (0-2hr) at 0°C; Reps = Number of replicates showing corresponding thermal event

Treatments included: All somatic embryos subject to loading solution (2M glycerol + 0.4M sucrose 1hr incubation at 0°C) ± PVS2 (0-2hr). Means ± SEM derived from 2-3 somatic embryos

Table 5.14 Bead/ explant water relations (3hr laminar air-flow desiccation)

Explant	Blank or Explant	% Sucrose in basal medium*	%Na-alginate in bead**	% RM***	OI (g/g DW) ****
Shoot-tip apex (ST)	Blank 	15 g /l	3%	52%	1.32
	ST 			55%	1.104
Somatic embryo (SE)	Blank 	30 g/l	3%	26%	0.539
	SE 			50%	1.108
ESM (No blank)	ESM 	30 g/l	5%	37%	0.479

*= basal medium sucrose content g/l

** = % (w/v) Na-alginate in bead

*** = % relative moisture of bead (% RM) results extracted from Tables 5.2 and 5.4

**** = Osmotically Inactive water (g/g DW) results extracted from Tables 5.2 and 5.4

This table 5.14 shows the relative effects of: (1) the type of explant and its bead volume occupation (visual approximation not drawn to scale), (2) the % sucrose in the corresponding liquid basal medium used to prepare the bead, (3) the % Na-alginate in the bead upon the % RM and the osmotically inactive (OI) water content after 3hr laminar air-flow desiccation.

5.4 Discussion

5.4.1 Shoot-tip apices

5.4.1.1 Encapsulation-dehydration

Summary

To circumvent lethal ice crystal formation in the shoot-tip apices of *P. sitchensis* it was necessary to eliminate all osmotically active water. This was best achieved through 0.75M sucrose dehydration followed by 3-4hr laminar air-flow desiccation, resulting in a water content of 31-63% (RM). Other pre-treatment factors including bead/apex size, cold hardening and DMSO significantly affected the water status and thermal stability. The optimal water content values in other woody plant species investigations report similar pre-treatment values. Ice nucleation/melt events were eliminated in olive tree shoot-tips following 2 days 0.75M sucrose pre-treatment, and laminar air-flow desiccation for 2hr reduced the %

RM to 30% (Martinez, *et al.*, 1999). This approach produced 30% survival in post-thaw shoot-tip apices. Encapsulated-dehydrated *Ribes* 'Ben More,' shoot-tip meristems showed ice event elimination and 60% recovery following 0.75M sucrose for 18hr after 4hr laminar air-flow desiccation (Benson, *et al.*, 1996).

The thermal profiles in encapsulated Sitka spruce shoot-tip apices corresponded well with similar studies using an identical cooling, warming and scanning rate on *Ribes sangineum* (Benson, *et al.*, 2005 In press). *Picea sitchensis* shoot-tip apex survival (20% of shoot-tip cryopreserved survived for 3 months) and re-growth was achieved following 1 wk of 4°C cold acclimation in 9% (w/v) sucrose, with or without 5% (v/v) DMSO, 4hr laminar air-flow desiccation and LN immersion, but degradation and senescence occurred after 3 months (see Chapter 4). The thermal profile for this set of treatments with 3hr and 4hr laminar air desiccation was very stable with no OA water and glass transition events occurring with or without the 5% (v/v) DMSO treatment (Tables 5.6 and 5.7).

Sucrose

In Sitka spruce apices, sucrose dehydrated beads subsequently desiccated for 0-3hr showed ice melt midpoints from –7 °C to 13 °C (enthalpy 10 to 140 J/g) and Tgs from -57°C to -19 °C. In comparison *R. sangineum* sucrose incubated beads desiccated for 0-3hr showed ice melts midpoints from -14 °C to 8 °C (enthalpy 13 to 200 J/g) and Tgs from -43 to -14 °C (Benson, *et al.*, 2005). One difference was observed in Sitka spruce encapsulated shoot-tip apices, ice melts and Tgs existed in the same sample, but in the *Ribes* samples these events were observed in separate samples; for instance ice melts were observed from 0-2hr air-desiccation and Tgs events after 3-4hr desiccation. The presence of both ice and glass transition phenomena in Sitka spruce encapsulated shoot-tip apices suggests that differential moisture gradients may have been formed in the alginate-tissue complexes during pre-treatment stages. Heterogeneity within the bead –explant system, appears to occur and may be reflected in different types of thermal events, some that may be assigned to the tissues and others to the encapsulation structures and treatment osmotica. Similar events have been reported to promote ice-nucleation or re-crystallisation during re-warming (Dumet, *et al.*, 2000). Cold hardening treatments, DMSO and sucrose pre-treatments applied to Sitka spruce shoot-tip apices may also have promoted similar gradients in alginate-tissue matrix.

Smaller more localised glass relaxation events or small minor thermal changes (termed localised glass transition events, LTgs, in this study) were observed (-20 to -50°C) during both cooling and warming cycles in both blank beads and beads incorporating shoot-tip

apices, but were less so following sucrose dehydration. These minor thermal steps were associated with both peak (nucleation/melt) and Tg events and their effect on thermal stability is unknown. Similar profiles and step magnitudes were observed in *R. sangineum* and following 24hr 0.75M sucrose pre-treatment in *Citrus sinensis* embryonic axes, where an endothermic step was observed at -35°C (Santos and Stushnoff, 2003). These events were not thought to influence the general stability of the bead structure as a whole.

OA/OI water

Shoot-tip apices not pre-treated with 5% (v/v) DMSO showed a reduction in the total water content to 31% RM following 3hr desiccation, resulting in ice elimination. The RM content of 3hr laminar air-flow desiccated *Ribes sangineum* was 23-25.7%RM, however these beads had an additional osmotic component having been loaded with 0.75M sucrose in the Na-alginate composition, as well as an 18hr 0.75M sucrose dehydration step (Benson, *et al.*, 2005 In press). *Picea sitchensis* shoot-tips with a 5% (v/v) DMSO pre-treatment always showed a greater RM from 63 to 42% for 3-4hr desiccation respectively. This appeared to be linked with the high amount of osmotically inactive (OI) water associated with shoot-tip apices that had been treated with DMSO.

Following dehydration and desiccation treatments Sitka spruce shoot-tip apices contained twice the OI water values of *R. sangineum.* These higher OI water values in Sitka spruce may be associated with the complex pre-treatment regimes and the modes of cold-hardening and DMSO interactions. Another feature of OI water in Sitka spruce encapsulated shoot-tip apices was an increase during 0-3hr desiccation, following its reduction at 4hr; an observation also seen in silica dried blank beads (Block, 2003).

The sucrose pre-treatment was essential to reduce the bead water content (% RM). In this study, without sucrose the untreated blank bead contained twice as much water (g/g DW) as the encapsulated shoot-tip apex. When sucrose dehydration was undertaken, the water content was reduced by more than 50% (g/g DW) in blank beads and compared to 7% (g/g DW) in encapsulated shoot-tip apices.

Desiccation for 3hr largely reduced the % RM differences between sucrose and non-sucrose treated beads, which suggested non-sucrose treated beads showed very rapid, and possibly detrimental rates of water loss during desiccation. This *P. sitchensis* study shows similarities with *R. sanguineum* DSC studies (Benson, *et al.*, 2005 In press) inclusion of sucrose during encapsulation causing a more rapid loss of water during the subsequent 20hr of sucrose

dehydration (step 1), but showing a slower rate of loss in the 2nd step during laminar-air flow desiccation (first 3hr) than beads without sucrose. There was a greater difference in bead sizes between replicates that had not been sucrose dehydrated than those dehydrated with sucrose, indicating that sucrose influenced the osmotic balance to regulate size.

The production of a stable Tg in a thermal profile is correlated with survival (Benson, *et al.*, 1996). In Sitka spruce, 3hr air-flow desiccation of sucrose pre-treated beads only produced Tgs that did not destabilise upon re-warming from a range of bead sizes. A 4hr air-flow desiccation of sucrose pre-treated beads did not produce Tgs. This thermally stable profile showed the OI water was also reduced. Osmotically inactive water that is thought to be essential for cell function upon re-warming, is a factor that will be discussed in section 5.4.3. The 4hr air-flow thermogram (Fig.5.6c and d) showed a characteristic endothermic gentle upward slope during cooling and conversely an exothermic slope down during re-warming. This 'sigmoid-like curve,' without re-crystallization is reported to be due to the differences in molar heat capacity between the frozen solution and the amorphous solid cryoprotective mixture (Dereuddre, *et al.*, 1991).

Impacts of cold hardening (ch) and DMSO

Cold hardening and DMSO pre-treatments were incorporated into Sitka spruce shoot-tip apex pre-treatment regimes; both influenced the water status and resultant thermal events. The exclusion of cold hardening (7 days in an alternating light/dark at temperature of 20/4 $\pm$ 1°C) in encapsulated shoot-tip apices showed an increase (10% g/g DW) in OA water (Table 5.2) when compared to fully cold hardened encapsulated shoot-tip apices. The exclusion of sucrose pre-treatment produced a similar trend as the exclusion of cold hardening.

These results correlate with other studies suggesting cold hardening induces an increase in intracellular solutes (mostly in sugars) to protect against freezing nucleation. There have been studies where the direct chemical/physical implications of cold hardening or acclimation have been interpreted using DSC or other thermo-analytical methods. Benson, *et al.*, (1996) determined that cold acclimation, over 7 days, did not influence the nucleation or melt characteristics of *Ribes* shoot-tip apices. *In vitro* cold hardening is useful in cryopreservation protocols, and has been incorporated successfully in several species such as hops, (Martinez and Revilla, 1998) and in pears (Reed, 1990).

In this study shoot-tip meristem sizes varied greatly (Fig. 4.3). The size differences are believed to be due to a general heterogeneity in the *P. sitchensis* shoot-tip cultures

investigated (see Chapter 4) and a differential response to 1-week cold hardening. These factors superimposed on one another may have resulted in a highly heterogeneous shoot-tip structure, which when examined by thermal analysis does not solely represent a cold hardening response. The consequences of this heterogeneity, and therefore lack of uniformity in encapsulated shoot tips, will be discussed in section 5.4.3.

The implications of 24hr 5% (v/v) DMSO pre-treatment on water status and thermal stability were considered. There were no notable thermogram differences between DMSO inclusion and exclusion (Figs 5.6a and 5.6.b), and profiles are comparable to the those of *R. nigrum* (Benson, *et al.*, 1996). However, Table 5.2 shows that when DMSO is excluded from the pre-treatment protocol, the mean % RM is reduced by 20%. Without DMSO the mean % RM is similar to the reported dehydration water content (25% RM) for *Ribes* (Benson, *et al.*, 1996). This suggests the increased water content observed in the Sitka spruce shoot-tip apex experiments may well be due to the DMSO pre-treatment influencing the OI water content. Increased OI water was imaged by NMR instrumentation during the freezing of periwinkle cells pre-treated with DMSO (Chen, *et al.*, 1984).

The thermal properties and hydroxyl group position of a compound comparative to DMSO, ethylene glycol, was reviewed by Baudot, *et al.*, (2004). When the methyl function is attached to the same carbon atom as a hydroxyl group the electron density shifts from the methyl group to the hydroxyl oxygen, making the oxygen a stronger base. The hydrogen bond, in which it engages, is further enhanced and the connecting water molecules are less free. A similar bond may occur between water molecules, DMSO methyl groups and lipid groups in the cell membrane, in this case the DMSO, glycerol, sucrose, ethylene glycol cocktail of PVS2 may have an effect on OI water content.

5.4.1.2 Plant Vitrification Solution 2

Summary

Ice nucleating/melt events in beads incorporating shoot-tip apices were eliminated following cold hardening, 5% DMSO (v/v), 2hr 0.4M sucrose and 2M glycerol treatment at 0°C and 2hr PVS2 treatment at 0°C resulting in a % RM of 80.87%. An examination of the literature indicates that this is the first time that encapsulated-PVS2 shoot-tips have been examined by thermal analysis. In this treatment, evidence of a stable glass formation/relaxation was observed, defined by the Tg in Fig. 5.4. The formation of a stable glassy state is a prerequisite for vitrification-based cryoprotection; (Benson, *et al.*, 1996, Benson, *et al.*, 2005 In press, Dereuddre, *et al.*, 1991, Dumet, *et al.*, 2000). The influencing factors, determined in this

experiment, were the glycerol/sucrose pre-treatment and incubation time of the PVS2 treatment. The small standard error margin (SEM) for the % RM between replicate beads suggests that during PVS2 treatment, bead size did not greatly influence the water content and associated thermal profiles. This may be associated with the PVS2–H_2O bonding characteristics (see section 5.4.3).

A variety of PVS2 parameters have been optimised for thermal stability. Garlic shoot-tips treated with 120 min of PVS2 treatment (56.5 % RM) continued to show ice events in some replicates (Kim, *et al.*, 2005) whilst *R. nigrum* non-encapsulated shoot-tip apices pre-treated through cold acclimation and 5% (v/v) DMSO prior to 20 min of PVS2 treatment on ice produced thermally stable profiles with no ice events and a Tg (Benson, *et al.*, 1996).

Thermal stability

Encapsulated Sitka spruce shoot-tip apices showed thermal stability, with one Tg at -50 °C following 2hr glycerol and sucrose treatment and 2 hr PVS2 treatment. The temperature of ice melt onset decreased through progressive treatments from -1.32°C (no sucrose, no PVS2) to -34.6°C (blank+glycerol+sucrose+PVS2 1hr). The uniform depression of melt temperature (Table 5.8) with increasing cryoprotectant exposure suggests that there is a trend. One law of thermodynamics, 'Raoult's Law,' may offer a physical-chemical explanation for this trend, although there are limited reports of its biological applications, (Shearwin and Winzor, 1990). The law states that the vapour pressure of a solution of a non-volatile solute is equal to the vapour pressure of the pure solvent at that temperature multiplied by its mole fraction. This means that the freezing (melting) point of the solute in a solution is lower than that of the pure solvent. If the solvent, is water, and an increasing number of solutes are combined with the solvent, as in increased PVS2 application, then the melting temperature is depressed.

Unlike the melting points, Tg events did not show such uniform correlation and were observed between -25 to -50°C in most thermal profiles regardless of the thermal history i.e. presence/absence of an ice (nucleation/melt) event. The thermal profile differences between the encapsulated Sitka shoot-tip apices and other comparable experiments may reflect the potential buffering capacity of the Na-alginate bead. Draget, *et al.*, (1988) showed alginate entrapment improves protection of cells against strong gradients as well as mechanical stresses. The bead may aid a more controlled diffusion of cryoprotectants into the cells.

The % RM of thermally stable *P. sitchensis* beads was 30 % greater than non-encapsulated garlic shoot-tips (Kim, *et al.*, 2005), although all this water was noted to be osmotically inactive. In *P. sitchensis*, the most notable change in thermal events and water content

through progressive treatment was following glycerol and sucrose treatment. The OA to OI ratio was effectively reversed from 2:0.2 to 0.7:2. (Table 5.3) accompanied by a 2/3 reduction in enthalpy and peak size. There was no post-LN recovery in encapsulated PVS2 vitrified Sitka spruce shoot tip apices (see Chapter 4) treated with 30 min to 2hr of PVS2 but thermal profiling (Table 5.9 and 5.10) indicated that no ice nucleation or melting occurred following 2hr of PVS2 and so the lack of shoot-tip recovery may be attributed to a non-cryogenic cause.

5.4.2 Somatic embryos/ESM

5.4.2.1 Encapsulation-dehydration

Ice nucleation and melt events were eliminated following 0.4M sorbitol treatment, encapsulation and 0.75M sucrose incubation for 18hr and (a) 3hr desiccation in somatic embryos (50-54% RM) or (b) 4hr desiccation in ESM (40.7% RM). This is the first time that thermal analysis has been used to investigate conifer somatic embryos and embryogenic suspensor mass (ESM) cryopreservation procedures.

Somatic embryos

Encapsulated somatic embryos of the tropical tree 'Neem' required 0.75M sucrose dehydration and 4hr desiccation (13.1% RM) to produce stable profiles (Benson, *et al.*, 2005 In press) while Sitka spruce somatic embryos required 0.75M sucrose dehydration and 3hr desiccation to produce thermal stability. A lower Tg onset was observed in Sitka spruce embryos (-83.7°C) than in Neem embryos (-53.9°C). This difference may be due to the increased Sitka spruce embryo size and water content (50% RM).

In one Sitka spruce encapsulated somatic embryo, following 1hr of air-flow desiccation, a 'shoulder' on the ice melt peak (Fig.5.5d) was formed. This may have been due to the embryo separating from the bead possibly during crimping and this phenomenon was not observed in control blank beads following the same treatment. An interpretation is that the large peak was the bead water and that the small sharp peak was water from the detached embryo; similar events have been observed in nematode freezing investigations (Wharton and Block, 1997). Vertucci, *et al.*, (1991), determined that a sharp peak at 0°C freezing proved lethal in embryonic axes of *Landolphia kirkii*. Minor localised thermal transition (LTgs) (endothermic up) were observed during cooling in Na-alginate encapsulated embryos at 1hr and 3hr following air-desiccation between -25 and -50°C on cooling and -25 and 0 °C on re-warming

(Fig. 5.5c and e). It is not known if these events are problematic for thermal stability and survival and requires a more detailed examination.

Encapsulation-dehydration methodology was used to achieve up to 20% post–LN embryogenic suspensor mass recovery and up to 100% non-embryogenic mass in somatic embryos (Chapter 3). A 3hr laminar air-flow desiccation was sufficient to eliminate water to circumvent ice formation and melting (Table 5.10 and 5.11).

ESM

The ESM suspended in alginate matrix showed uni cellular and cell cluster thermal behaviour as cells developed into proembryos. Cell-cell and cell-surface interactions may play a significant role in the low-temperature response of tissue systems and in intracellular ice–formation (Acker, *et al.*, 1999). In tissue models cells in suspension froze at a lower temperature (-9°C) than cells in spheroids (-7°C).

DSC analysis on the ESM was limited to a 3hr and 4hr desiccation pre-treatment. A 5% (w/v) Na-alginate matrix was used to embed the partially dedifferentiated ESM cells. After 3hr desiccation, the blank control and encapsulated ESM showed an almost identical ice nucleation (-25°C), Tg formation (-70°C), ice melt (-10°C) and Tg relaxation (-45°C). Following 4hr of desiccation, just one Tg relaxation was formed at around -108°C. DSC examination of encapsulated *A. thaliana* suspension cells showed that a 7-day 1M sucrose liquid incubation followed by a 4hr silica gel dehydration (34% RM) produced the highest glass transition temperatures and the smallest ice crystallization events, correlated with the increased stability of the vitreous state, better survival and re-growth (Bachiri, *et al.*, 2000). In order for ice nucleation to be prevented a % RM of 50 % or less was required for somatic embryos and 40% RM or less for ESM.

There is evidence that *P. sitchensis* suspensor cells are highly vacuolated (Chapter 2) and this may explain why a 4hr desiccation time was required to remove all osmotically active water from the cells (Table 5.4). Bachiri, *et al.,* (2000), undertook ultrastructural studies on *A. thaliana* suspension cells after sucrose pre-treatment and determined that a 1M sucrose pre-culture for 7 days reduced the vacuolar volume by redistribution of the large vacuoles into a number of smaller ones. Bachiri, *et al.,* (2000), also determined that after 4hr desiccation (34% RM) some crystallisation on re-warming was occurring but cell survival was greater after 3hr or 5hr desiccation. Chen, *et al.*, (1984) also determined, using nuclear magnetic resonance and differential thermal analysis, that there was a close association between the per

cent of unfrozen water at -40°C and survival in DMSO and sorbitol pre-treated *C. roseus* (L).G. Don cells. A similar sucrose pre-treatment may assist better dehydration of Sitka spruce suspension cells to prepare them for desiccation and rapid cooling, but this requires further investigation.

Thermal analysis demonstrated that a minimum of 4hr laminar air-flow desiccation was required to ensure no ice was formed during cooling or upon re-warming (Tables 5.10 and 5.11). However, because of the desiccation sensitivity of the encapsulated ESM (see Table 2.4 in Chapter 2), a laminar air-desiccation time of 2hr was selected for ESM cryopreservation survival trials. The limited post-LN recovery (20% in 1 of 5 genotypes tested, Fig. 2.9 Chapter 2) is likely to be in part due to a sub-optimal desiccation phase resulting in the formation of lethal intracellular ice.

5.4.2.2 Plant Vitrification Solution 2 (naked somatic embryos)

Ice nucleation and melt events were not observed in naked embryos following 0.4M sorbitol pre-culture, a 0.4M sucrose and 2M glycerol osmoprotection at 0°C for 60min and 30min of PVS2 incubation at 0°C and ice elimination was achieved at a RM of =<16.7% (water content; 0.2 g/g DW), all of which was OI water. This is the first time that thermal analysis has been undertaken on naked somatic embryos during PVS2 treatment. This analysis revealed that the naked embryos behaved very differently to those that were encapsulated.

Ice nucleation and melts were observed in the somatic embryo following sorbitol pre-treatment only with a 32.5% RM. A 30 min PVS2 treatment produced thermal stability with no Tg on cooling and a thermal event on re-warming which may be a Tg or a destabilisation event. The Tg temperature following 1hr of PVS2 was depressed to –130°C during cooling, and glass relaxation did not occur until -30°C on re-warming.

The thermal profiles were considerably complex considering the comparative simplicity of the explant and sorbitol pre-culture without other treatments. Even without PVS2 treatment three separate thermal events were observed during cooling (Fig. 5.7). Firstly, a relatively small but significant nucleation event (65.43±21.85 J/g) when compared to the enthalpy of an encapsulated embryo (164.9 ±15 J/g), occured at 25 to 0°C. Secondly, of lesser importance, localised Tg, seen on all thermograms at -50 to -75°C and more importantly, thirdly a Tg at -120 to -175°C. When PVS2 treatment was increased the ice peaks disappeared and what looked like a second Tg replaced it. The localised Tg may be related to the sorbitol treatment.

This hypothesis could be tested by determining a DSC profile of an untreated embryo, but requires a more detailed examination.

The naked embryos contain the lowest water content RM (30-10%) 0.8 to 0.1 g/g DW in this experiment. Thirty min PVS2 treatment reduced the water content 4-fold (0.2 g/g DW); increasing PVS2 exposure to 30 min more, halved the water content again (0.1 g/g DW). In embryonic axes, excised from *L. kirkii,* melting transitions were not observed between 0.29 and 0.22 H_2O g/g DW (Vertucci, *et al.*, 1991). In Sitka spruce embryos, the water content was reduced after 1 hr PVS2 treatment to an osmotically inactive form.

The cryopreservation of an explant without a Na-alginate bead has revealed events that appear to have been masked or stabilised when a bead is present. However, not all explants would tolerate manual manipulation and direct osmotic stress without the protective bead exterior. The thermal analysis investigation and post-LN somatic embryo recovery trials (Chapter 3) correlated very well. Following osmoprotection, the non-encapsulated somatic embryos required only 30 min of PVS2 vitrification to ensure ice was eliminated and stable glass transitions formed (Tables 5.12 and 5.13). In post-LN recovery trials, somatic embryos showed up to 100% recovery in the form of non embryogenic masses (NEM) when the 30 min PVS2 treatment was applied (Fig. 3.15, Chapter 3).

5.4.3 Critical factors for all explants

5.4.3.1 Importance of osmotically inactive water to cellular functions

There are several examples throughout the Sitka spruce explants and pre-treatment cryoprotection regimes, where the presence of osmotically inactive water is highlighted. DMSO, sucrose and cold hardening all increased the osmotically inactive water content, in shoot-tip apices, whereas OI water decreased during desiccation until a threshold was reached.

Table 5.14 shows that encapsulated shoot-tip apices and their equivalent blank beads show similar water contents, but encapsulated somatic embryos contain twice as much water (g/g DW) as their blank equivalents. Inclusion of a shoot-tip apex or somatic embryo explant creates beads with very similar % RM and OI water contents (1.104 and 1.108 g/g DW) despite differences in the equivalent blank bead % RM and OI contents. This may suggest that there is more OI water in the somatic embryos than the shoot-tip apices. The water content of beads including ESM was half that of other encapsulated explants (0.479 g/g DW).

It is suggested that the somatic embryo bead matrix contained less water because of the dehydration effect of the higher sugar content in the liquid medium used to prepare the Na-alginate. Somatic embryo and shoot-tip apex explants were approximately the same size so the somatic embryo without the bead must have contained more OI water than the shoot-tip apice. The ESM with a bead contained the least % water FW.

The variability in OI water content was a critical factor for successful cryopreservation and may be associated with lipid content. Dussert, *et al.*, (2001) showed that decreased OI water content was correlated with increased lipid content in the endosperm of seven coffee species seeds. Lipid contents, in several conifer explant types have been reported. Bornman, *et al.*, (2003) suggested that because in *P. abies* >80% of protein, carbon and amino acid reserves are accessed from the megagametophyte, zygotic and corresponding somatic embryos would not have high intercellular reserves. In *P. glauca* engelmanni increased lipid content was observed through somatic embryo maturation and ABA stimulation (Carrier, *et al.*, 1997). In *Prunus avium*, the lipid content in mature somatic embryos increased substantially following cold treatment; lipid bodies surrounded by protein bodies were observed in cotyledonary cells (Reidiboym-Talleux, *et al.*, 2000).

In consideration of the literature (Bornman, *et al.*, 2003) and the water contents determined for each explant it appears that the somatic embryos may contain less lipid and therefore have a correspondingly high OI water content and the cold-hardened shoot-tip apices consisting of meristematic cells contain more lipid with a smaller amount of OI water. Without the corresponding blank bead for encapsulated ESM it is difficult to interpret its low water status, discussed in Chapter 2. ESM consists of simple highly vacuolated suspension cells that are likely to have high OA water contents, thereby enhancing the effects of osmotic gradients. Total lipid content can be measured using a simple solvent extraction (Folch, *et al.*, 1957) and assist in these investigations.

In this study, both the shoot-tip apex and somatic embryo studies of PVS2 treatments were associated with high % RM and % OI water. Two reasons for this effect may be (1) cryoprotectants entering the cell and interacting with water to prevent its loss during dehydration and prevent its freezing, and/or (2) the % RM may be overestimated by the volatile components during drying at 105°C. The RM % of naked garlic shoot-tips during vitrification with PVS2 (Kim, *et al.*, 2004) and PVS3 (Kim, *et al.*, 2005) has been investigated and RM was calculated in a comparable manner. Over estimation by volatile components may therefore be ignored in this comparison.

Some cryoprotectants, in particular DMSO, can enter the cell and interact with H_2O. The RM % was strongly negatively correlated to the DMSO (PVS2), sucrose and glycerol (PVS3) concentrations. It was also determined that regardless of the pre-treatment the RM following PVS2 was similar, between 54.8 and 57.4%. In encapsulated *P. sitchensis* shoot-tip apices RM varied between 80.8 and 95.4%; in naked somatic embryos the RM was lower at 10-16%. It is theorised that the *P. sitchensis* shoot-tip apex, Na-alginate beads may have reduced the initial rapid penetration of PVS2 components until a 2hr incubation was applied and consequently the RM remained high compared to non-encapsulated garlic shoot-tips. In *P. sitchensis* DMSO was able to penetrate the non-encapsulated somatic embryos more easily and RM % was subsequently reduced. Kim, *et al* (2004) also noted that there were delays in the DMSO penetration into shoot-tip core regions compared to the periphery; such physiological contrasting cell zones would not be present in somatic embryos. Further work is required to examine PVS2 and PVS3 component penetration in varying physiological explant types.

The interaction of membranes, proteins, H_2O and DMSO, and the mechanisms by which water may become less available for freezing are unknown. Crowe, *et al.*, (1990) proposed that DMSO serves as a cryoprotectant of proteins and stabilizes phospholipid bilayers. The mode of interaction is at low temperatures at which hydrogen bonds are strongest and hydrophobic interactions weakest, allowing DMSO to be preferentially excluded from protein and hence serving as a cryoprotectant. There appears to be a gap in the literature in plant membrane DMSO-H_2O interactions and further studies are required to understand the underlying mechanisms.

There are numerous reports regarding the consequences of the removal of OI water upon survival. Sun, (1999) reported damage, assessed by electrolyte leakage, to acorns when the water content was reduced below 0.3g per gram dry weight. These consequences were summarised by Mazur, (2004). Loss of this water may affect enzyme/substrate reactions causing denaturation and cause damage to membranes in the form of fusion. Inactive water is thought to be important for cell function upon recovery. Some plant systems may survive limited ice formation if only small crystals are formed as the complete removal of osmotically active water is detrimental to survival (Benson, *et al.*, 2005 In press).

5.4.3.2 Thermogram profile anomalies/complexity phenomena

Minor thermal glass transition or relaxation events (termed, LTgs in this study) were observed in these DSC thermal profiles and in other reported DSC systems (Stushnoff, *et al.*, 1992). It

is unlikely that these small magnitude, endothermic events would disrupt the thermodynamic equilibrium as they absorb energy and may even contribute towards stability. A modulating DSC (Wang and Haymet, 1998), which is able to separate complex transitions, may assist in clarification of these anomalies. In some thermograms (Fig. 3.5e and f) no mobility or Tgs were observed. The non-detection of Tgs may indicate the limit of instrumental sensitivity but still confirm vitrification as no nucleation or melt events were observed.

5.4.4 Genebank applications

5.4.4.1 Bead and apice size

In encapsulated-dehydrated shoot-tip apices, there were differences in the thermal event profiles between replicates of the same treatment group [Table 5.6 st-suc+ch+DM 3hr desiccation; ICE (3/3), TG (2/3)]. The diameter of the pipette tip used to drop the Na–alginate into $CaCl_2$ solution to allow polymerisation was regulated, so that a bead of <4mm (volume ca. 50µl) was produced. The weight was influenced by the size of the shoot-tip apex.

Practical Considerations

The standard shoot-tip apices were reduced to a size where 4 individual primordia surrounded the apical dome; the crown was left intact as described Chapter 4. In 'dormant' shoot-tip apices, primordia were tightly packed against the apical dome, and the apex could not be further reduced without causing damage to the dome cells, hence they were larger in size. In some instances, 1 week cold hardening induced this response, but it was unpredictable and therefore of limited use. This may explain, why there was less variability between replicates in the treatment group that excluded cold hardening. Other studies have also shown correlations between *in vitro* hormone treatment and explant size differences. Kim, *et al.*, (2005) determined that the fresh weight of garlic shoot-tips was significantly higher when they were pre-cultured in 0.3mg/l zeatin and gibberelic acid.

The greatest variability between replicates was observed in beads in the absence of sucrose during pre-treatment. It is hypothesized, that the 0.75M sucrose incubation over 18hr dehydrated all beads to the point of osmotic equilibrium to produce uniformity of size. When shoot-tip apices were treated with PVS2 the bead size did not influence the water content so markedly (Table 5.3). It is thought that the osmotic potential of these solutions may further regulate the size of the bead through sucrose dehydration. A size range of garlic shoot-tips treated with PVS3, was examined through thermal analysis. Size was not found to

significantly influence thermal behaviour, but the larger apices (4.5x3.5mm) showed higher enthalpies compared to the smaller ones and contained a lower concentration of glycerol and sucrose (Kim, *et al.*, 2005).

5.4.4.2 Preparation for DSC analysis

Critical factors for bead preparation included bead composition and Na–alginate concentration, desiccation time and operation time (time in transferring beads to the DSC). These factors parallel those established by Block, (2003) who examined blank bead moisture content and thermal profiles.

Firstly, the bead composition and Na-alginate concentration was optimised for *in vitro* maintenance for each explant. For example, shoot-tip blank beads contained more water (% RM) than somatic embryo blank beads relating to the increased sucrose and Na-alginate in shoot-tip beads. However all explant/bead groups followed a similar pattern during desiccation regardless of explant. There were 3 identifiable stages: (1) reduction of ice nucleation/melt peaks, (2) Tg formation at around -40°C and (3) 'sigmoid-like curve' during cool/warm cycles and very low temperature Tg around -100°C often during the melt. These differences highlight that *in vitro* and cryopreservation protocols need to complement each other for a successful cryopreservation programme.

The second factor, highlighted in this study was that the temperature and relative humidity of the area around the DSC equipment and laminar air-flow bench needs to be regulated to ensure bead desiccation uniformity. A small difference in RH alters the desiccation requirements to produce the optimal water content before immersion in LN and DSC cooling. In an environment, where this is difficult or impossible (as in a laboratory without air-conditioning) silica gel may be used, as Sherlock, *et al.*, (2005) proposed. To desiccate, *Ribes* species to a critical point (0.4g/g DW) where stable glasses were known to form a 5hr desiccation at 16°C was required. The volume of silica gel and its activation state need to be optimised to achieve these desired water contents.

Thirdly, careful experimental planning is required to ensure uniformity in bead water content and thermal profile repeatability. Planning was required to stagger the treatments so that no bead was kept more than 30min between crimping and cooling, as the beads (especially, non-desiccated beads) were sensitive to changes even after sealing in aluminium pans. Similar concerns are also bead preparation, polymerisation and alginate content (Block, 2003).

5.4.4.3 Correlations between DSC analysis and explant survival

DSC has proven to be an effective tool in assisting in the optimisation of a range of vitrification–based cryopreservation protocols: encapsulation-dehydration, encapsulation-vitrification (PVS2) and PVS2-vitrification optimised for three explants of *P. sitchensis* shoot-tip apices, ESM and partially matured somatic embryos.

In encapsulation-dehydration protocols, thermal analysis showed that in all explants sucrose dehydration and a 3hr laminar air-flow desiccation was essential to eliminate ice nucleation. With the exception of encapsulated ESM, a 4hr desiccation time was not required to circumvent ice nucleation. In both shoot tip apices and somatic embryos protocols designed on optimal post-LN survival/recovery correlated well with thermal analytical stability. This was not the case with encapsulated ESM, but the reason for low post-LN survival, may be explained by the ice nucleation and melt peaks observed during DSC. The difficulties in balancing desiccation and thermal stability in ESM would indicate that an alternative cryopreservation method not based on desiccation may be more suitable for this explant.

Encapsulation-vitrification (PVS2) and PVS2-vitrifcation thermal analyses assisted in pinpointing the shortest PVS2 vitrification time necessary to circumvent ice nucleation and produce a stable thermal profile. This was explant and encapsulation dependent. Non-encapsulated somatic embryos required only 30 min PVS2 vitrification, whereas encapsulated shoot-tip apices required 2hr. Although no survival was observed following encapsulation-vitrification of shoot-tip apices, in this investigation, there is a protocol available with an analytical basis (not only empirical) that may be applied to other, younger more vigorous *P. sitchensis* cultures in future studies.

5.5 Conclusions

- Thermal analysis was undertaken on three explants following previously optimised pre-treatment regimes (Chapter 2-4).
- The aim was to determine the optimum parameters for each explant/cryopreservation protocol to achieve thermal stability and the elimination of damaging ice events.
- Post-LN survival and thermal stability without ice events correlated well where survival data was available (somatic embryos) and in other explants a great deal of information pinpointing water contents in relation to osmotica pre-treatments, laminar air-flow desiccation requirements and vitrification treatment exposure was obtained.

- Water decreased progressively with dehydration/desiccation treatments.
- Many of the thermograms were complex, especially those with PVS2 treatment. Thermal behaviour is influenced by the water content, pre-treatment history prior to glass formation, and the complexity and heterogeneity of glassy matrices and their cellular components. Ice nucleation was eliminated in all cryoprotective strategies of optimised pre-treatments to produce stable thermal profiles. The profiles produced correlate with other species where post-cryopreservation survival has occurred. This suggests that these protocols are thermodynamically stable and that other factors are responsible for explant survival.
- Under optimum pre-treatment regimes, osmotically active water was completely removed but the relationship of osmotically inactive water to survival and biological interpretation is not yet fully understood. The more complex cell composition of the shoot-tip apices and consequent differential penetration and dehydration modes of sugar and cryoprotectant treatment may explain why shoot-tips were so recalcitrant to cryopreservation. Further work is necessary to assign individual components to specific thermal events, and this was not within the scope of this study.
- Na-alginate encapsulated explants showed similar thermal profiles. Following encapsulation-dehydration, blank beads and encapsulated shoot-tip apices/somatic embryos generally behaved in a similar fashion throughout progressive desiccation. Three stages were characterised: (1) large ice nucleation and melt peaks (enthalpy 250-80 J/g) after 0-2hr, (2) smaller ice-nucleation/melt peaks in encapsulated shoot-tips and embryos, mostly not in blank beads, after 3hr with Tgs, (3) no obvious events on a curved line showing thermal stability after 4hr. Somatic embryos showed greater OI water than shoot-tip apices, and further work must be undertaken to determine, if this difference is due to variability in lipid content due to explant physiology and/or pre-treatment stress (cold-hardening).
- In encapsulation-dehydration treatments bead size was an important factor, although sucrose dehydration assured some degree of homogeneity, further controls might be required to assure precise rates of desiccation. PVS2 treated beads showed less variability in total water and OI water between replicates and where bead size was not so critical. Naked somatic embryos required a simpler PVS2 treatment compared to encapsulated explants.
- In conclusion these studies focus attention to control relative humidity, bead size and transfer timing between operations, as water status is very sensitive to these critical factors.

Chapter 6 APPLICATION RECOMMENDATIONS AND TECHNOLOGY TRANSFER IMPLEMENTATION

6.1 Introduction

The applications goal of this project was to develop cryopreservation protocols for *in vitro* germplasm of Sitka spruce. The project concludes with the completion of recommendations to the Northern Research Station (NRS) related to the implementation of cryostorage technology by the Forestry Commission. Training provided at institutions gained from two travel awards and affiliation with the European Commission's COBRA and CRYMCEPT cryopreservation projects assisted in the development of technology transfer recommendations. A study tour of COBRA research institutes in the Czech Republic (Society for Low Temperature Biology's Audrey Smith Travel Award May 2002) was undertaken to assist in the development of cryopreservation methods. Three cryobanks were visited; the Institute of Soil Biology SAS in Ceske Budejovice, the Institute of Botany in Trebon and the Research Institute of Crop Production, Prague. A visit to the Plant Physiology Department at the Charles University, Prague demonstrated current conifer somatic embryo research (Svobodova, *et al.*, 1999) that later underpinned the development of cryopreservation methodology for *P. sitchensis* somatic embryos at UAD.

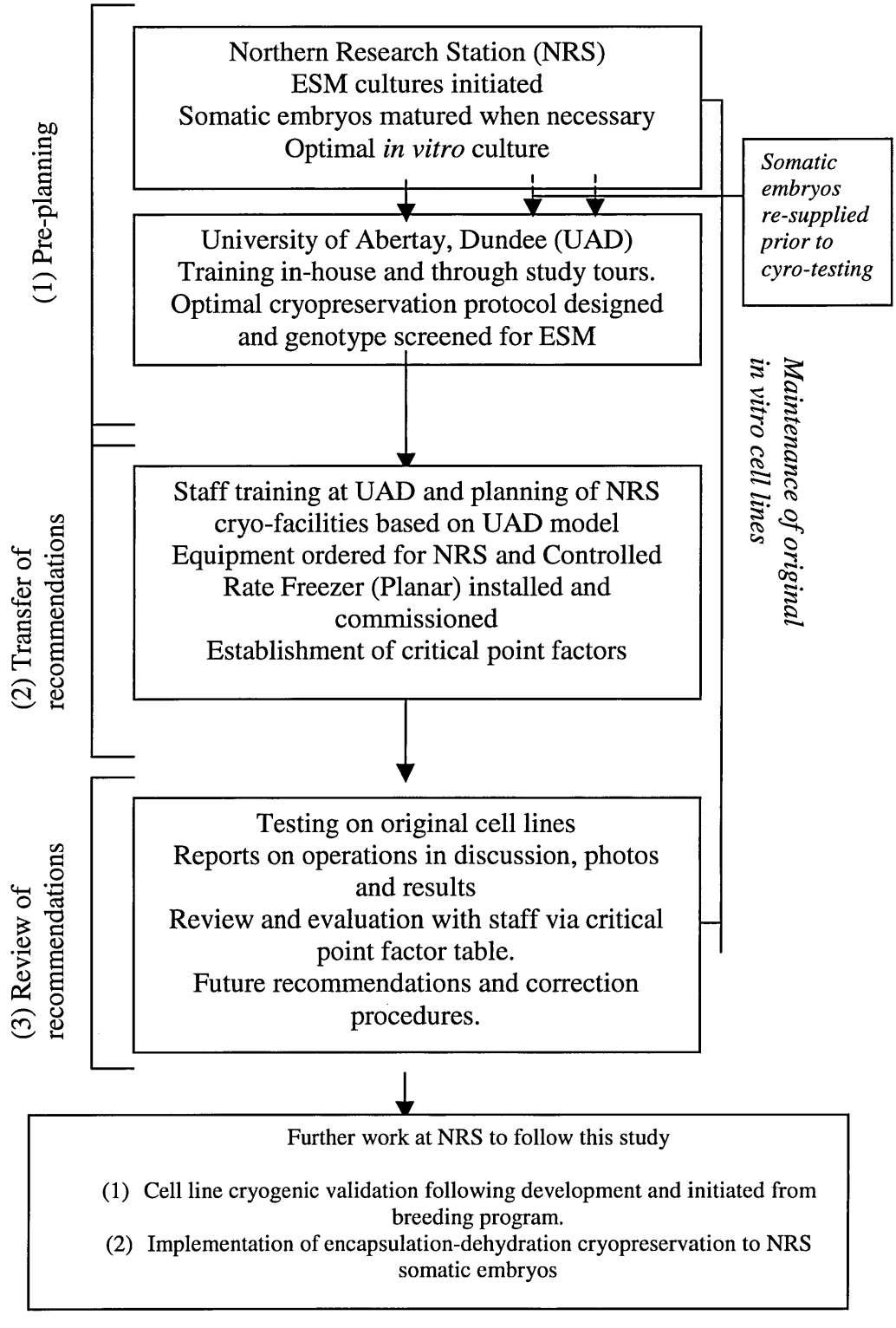
A study tour (Scottish International Education Trust (SIET), October 2003) was undertaken to several Forest Research Institutes in Canada. Practical ESM cryopreservation training was undertaken with the Canadian Forest Service - Maritimes Region at Natural Resources Canada, Fredericton in New Brunswick. New methodologies produced a 99 % success rate achieved during cryopreservation (Park, *et al.*, 1998, Park, *et al.*, 1993, 1994) in all but one of the native *Picea* spp. in Canada. A study tour to the Plant Biology Group at the University of Calgary, Alberta, under the direction of leading somatic embryo expert, Professor Trevor Thorpe, (Thorpe, 2000, 2004) inspired modifications to original protocols and recommendations during the technology transfer to NRS. This chapter therefore presents the first stage (within the time–frame of the study) of technology transfer.

6.1.1 Planning and strategy for technology transfer of ESM and embryos

The planning and development of technology transfer was implemented in three stages (Figure 6.1). During the three stages original ESM cultures were maintained *in vitro* through serial subculture at NRS as a core collection, back-up, a source of somatic embryos and for comparison with recovered post-cryogenic ESM. Firstly, pre-planning consisted of transferring ESM cultures to NRS, ensuring *in vitro* growth characteristics were comparable to those observed at NRS, undergoing international and in-house training in cryopreservation techniques and using these techniques to develop safety procedures, cryopreservation facilities, protocols and record keeping systems that were feasible for implementation at NRS.

Secondly, cryogenic facilities were installed and training provided for NRS staff. Thirdly, cryopreservation at NRS was undertaken and validation progress was reported to the UAD trainers using critical point factors from which future recommendations and corrective procedures will be undertaken beyond the timescale of the project.

Figure 6.1 The phases of technology development and transfer in the cryopreservation of *Picea sitchensis* germplasm from research institute UAD to collaborative forestry institute NRS.



ESM=Embryogenic suspensor masses
UAD=University of Abertay Dundee
NRS=Northern Research Station

6.1.2 COSHH and Health and Safety

COSHH (Control Of Substances Hazardous To Health) became law on 1st October 1989, with a new version in 1994, with the purpose to set down efficient step-by-step approaches to control hazardous substances and to protect employees from exposure to them. Failure to comply is an offence under the Health and Safety at Work Act 1974 and the COSHH regulations. COSHH forms are required for operations using liquid nitrogen.

For all cryogenic manipulations a risk assessment must be performed in collaboration with the local or in-house safety officer. Risk assessment forms must be completed for the use, handling and transport of liquid nitrogen and for controlled rate cooling apparatus which run the particular risk of a sudden pressure release and LN exposure. At -196°C LN can cause cryogenic burns and frostbite on contact with skin and eyes and one litre of liquid nitrogen vaporises into almost 700 litres of gas that may cause asphyxiation in a confined, poorly ventilated environment. It is for this reason that cryogenic protective clothing including, goggles, a full-face shield and heavily insulated gloves and non-open shoes must be worn when using or handling LN. LN users should be consequently trained to handle LN and know the emergency procedure if the oxygen monitor /alarm is triggered. During rapid vaporization of LN cryovials sometimes explode and users must employ careful handling with full cryo-protective clothing to prevent serious penetration injuries. LN should ideally be stored on the ground floor where no elevator is required.

Cryoprotective substances commonly used in cryopreservation procedures such as the cryoprotectants must be subject to COSHH and risk assessment. DMSO for example is categorized in a medium hazard class because it is a putative teratogen. Consequently current control measures for this substance stipulate that only short term (<10 min) procedures should be undertaken on an open bench, or within a fume cupboard and that gloves must be worn.

6.1.3 Design of a cryopreservation facility

Cooling apparatus and LN storage requires maintenance in a secure, clean and stable environment with appropriate inventorial documentation.
The programmable freezer and the Dewars should be maintained in:
(1) a well ventilated area, to avoid oxygen depletion during filling or spillage
(2) a dedicated room preferably with restricted access to authorised personnel only for security and to avoid general environmental microbial contamination

The room should also contain:
(1) documented emergency and health and safety procedures
(2) oxygen-depletion alarms (visible and audible) located below head height and in a large facility alarm should be linked to extraction fans
(3) an inventory of all stored material, withdrawals and entries
(4) low LN and high temperature warning alarms inside the Dewars

6.1.4 Operations in a cryopreservation facility

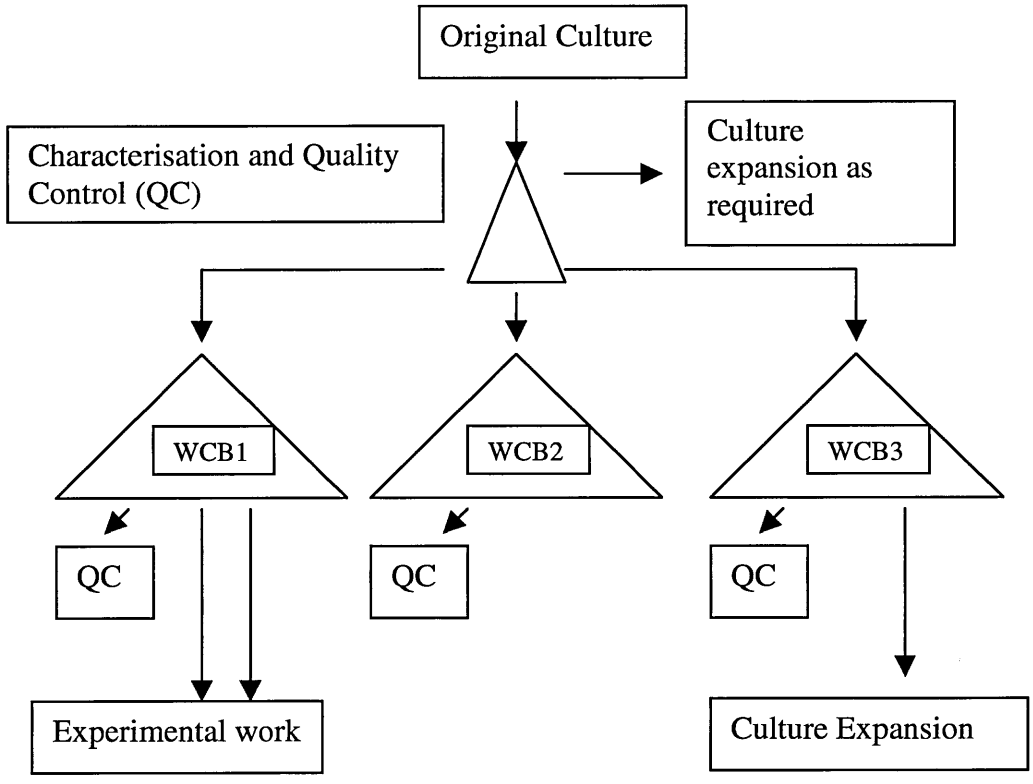
A model of, 'good practice,' in a working cell bank system is shown in Figure 6.2. Following the expansion of the initial culture at an early stage, germplasm is cryopreserved and transferred to Working Cell Banks (WCB), where quality and contamination controls are undertaken on a regular basis. Germplasm may be removed from the WCB 1 and WCB 3 for utilisation and multiplication respectively, but WCB 2 is maintained as the back-up Dewar. Diagnostic methodology and predictive measures may also be applied to: (1) determine the germplasm longevity and expected biological changes in liquid nitrogen storage (-120 to –196 °C) over time (Walters, *et al.*, 2004), and (2) the amount of germplasm required to ensure minimum survival (Dussert, *et al.*, 2003).

6.1.5 Cryopreservation

6.1.5.1 Skills transfer

Development of standard techniques for cryopreservation is vital for the global plant conservation community and as more isolated facilities are established a standard format for inter-institute training may also be preferential. Reed, *et al.*, (2004) have established a system of evaluation by critical points during technology transfer of plant cryopreservation procedures. A two-week training workshop in cryopreservation methods, in which investigators from several countries participated was undertaken.

Figure 6.2 Workings of master and cell banks (Stacey, 2004).



WCB =Working Cell Banks

After one-year critical points in the procedures were identified by the technology trainer visits to the reciprocating laboratory. Critical points were broadly identified as:

(1) Cryogenic (cryoprotection, re-warming)

(2) Non-cryogenic (plant health status, pre- and post-storage)

(3) Operational (skills transfer, training, interpretation of procedures)

(4) Facility (growth room and ambient conditions, media preparation, equipment differences)

Supplementary training was provided, where required, followed by a second visit by trainers. *Ribes* shoot-tip survival increased over the duration of the project.

6.1.5.2 Technology transfer

Critical factors in technology transfer include, cell bank composition (cryopreservation may select for atypical cell types); quality control testing (cell bank samples to provide information on purity, authenticity and microbial contamination); low-temperature stability (temperatures below -100°C must be maintained to avoid physical changes); storage duplication (duplication of genotypes at more than one site); the practise of asceptic technique and documented records (culture and quality control documentation) (Stacey, *et al.,* 1999).

6.1.6 Cryogenic Operations

a) Culture selection

The number of replicate cryovials per accession per genotype must be determined during preliminary cryopreservation optimisation. The number of cells preserved in individual cryovials must demonstrate sufficient competence to regenerate cultures that reproduce the characteristics of the parental culture (Dussert, *et al.*, 2003, Stacey, 2004).

b) Cryopreservation procedures

Procedure and protocols should be developed and optimised to the feasibility parameters of the recipient facility. These parameters will assist in the determination of cryogenic factors such as explant type (e.g. shoot-tip apex, somatic embryo, dedifferentiated culture), cooling technology and applicable methodology (high or low-cost technology) and staff training requirements (e.g. more intensive for shoot-tip dissection).

c) Re-warming and recovery

As with pre-cryo procedures, post-cryopreservation factors need to be undertaken in a clean and preferably dedicated environment. If heated water baths are required for re-warming care must be taken so as to avoid contaminated water entering cryovials (through use of a polystyrene float or sterile water container). The exterior of the cryovials should also be sterilised with IMS (Industrial Methylated Spirits) or an equivalent sterilising agent.

d) Quality control

Quality controls ensure that the cell line is viable, complies with a number of predetermined characterisation markers and that it is not contaminated.

Characterisation

Investigations of cytogenetics, morphology and biochemical function may be used to chracterise the cultures.

Contamination detection

Cell cultures can support the growth of bacteria and fungi that overwhelm the cells and lead to cell death, or are more subtly affected due to mycoplasma that are associated with the cell membrane. The mycoplasma are smaller than most bacteria and their presence may not be immediately detectable. The cell-doubling times of microrganisms is very short (2hr) and trace contamination may overwhelm cryopreserved cultures during recovery (Stacey, 2004). There are a number of methods available to screen and index contaminants in plant tissue and cell culture including:

(1) Transfer plant material to liquid or solidified sterility test media (usually based on meat or plant extracts) such as MS medium supplemented with peptone and yeast extract (Leifert and Waites, 1990) and maintained at 20-25°C, 3-7 days in a quarantined culture area to avoid contamination spread to core and working culture collections.

(2) Biochemical tests such as Gram stain, motility, gelatinase, oxidase and oxidation/fermentation (Reed and Tansprasert, 1995). The isolation and identification of the plant pathogen may be useful to identify the source.

Viability testing methods include:

(1) tetrazolium triphenyl chloride (TTC) – dehydrogenases and NADH reduce TTC forming a red formazan product that can be detected spectroscopically, (Steponkus and Lanphear, 1967).

(2) fluorescein diacetate assays (FDA) the diacetate is split by active membrane esterases releasing fluorescein, which cannot pass through the membrane. In viable cells the fluorescence is trapped and concentrated and may be observed under UV light (Widholm, 1972).

6.1.7 Records, cryovial/cane identification and documentation

It is essential that accurate records of stored germplasm are maintained so that cryovials may be retrieved quickly and efficiently without compromising the stability of surrounding germplasm accessions (Walters, *et al.*, 2004). In cell lines of genetically modified, infectious or hazardous material this may be a legal requirement. Records should be available both on

shared computer files in databases such as MS Access and in hard-copy format. Important reference numbers include the initial cell-line accession number for that culture, a cell bank reference number to specify cryopreservation and accession batch and if necessary media and quality control related references.

6.2 Transfer of recommendations

The aims of this stage of the project were:

(1) To prepare both staff and facilities at NRS for cryopreservation protocols through design meetings, visits and training
(2) To transfer cryopreservation protocols for Sitka spruce ESM and somatic embryos

6.2.1 COSHH, Health and Safety and Quality Assurance

All the COSHH and risk assessments at all stages of the process were implemented by NRS prior to cryopreservation testing. An additional Quality Assurance system was also implemented, at the insistence of the Department for Environment Food and Rural Affairs (DEFRA), to demonstrate that the facility is operating to a particular quality standard, fulfilling legal and technical obligations.

Standard operating procedures were written and implemented. It was recommended that NRS implement a policy similar to UAD in filing hard-copies of COSHH and risk assessment both in the laboratory and on a shared Excel file maintained on the C-drive on shared access computer. An oxygen depletion alarm was installed in the LN Dewar storage facility and protective cryogenic clothing was purchased.

6.2.2 Implementation of a cryopreservation facility

Cooling

The NRS Planar Kryo 560-16 Programmable Freezer with an MRV controller was housed in the main *in vitro* laboratory (Figure 6.3b). An extraction fan was fitted for the required number of air changes. A low oxygen monitor was also installed below sitting and standing head height.

LN storage area

A designated ground floor space was assigned for two 40–litre LN storage Dewars (wide necked, MVExc 47/11-10) housing cryo-baskets. One Dewar is a master storage Dewar, and one is a working storage Dewar. In addition two 30-litre LN Dewars (MVE Lab 30) are in place, one for LN storage and the other for dispensing and for the programmable freezer. The

area was easily accessible; 50 m from the LN delivery point, 50 m from the laboratory (where the new Planar Kryo 560-16 Programmable Freezer with an MRV controller was installed) and next door to the culture transfer room (housing the laminar air flow–bench where encapsulation-based procedures could be undertaken).

Storage Dewars are re-filled by two members of staff with assistance from a specially designed cradle (see Fig. 6.3a cryobaskets and nylon webbing for securing Dewar in pouring frame). The cradle is rotated and the height adjusted so LN could easily be poured from another Dewar to small Dewars. The LN storage Dewar room did not contain a low oxygen alarm and was constantly vented to the out side, but was sealed from outside environmental contamination. Low LN warning alarms (Fig. 6.3a) were installed for the storage Dewars. The floor is resin sealed concrete (Fig. 6.3a) to prevent damage in case of LN spillage.

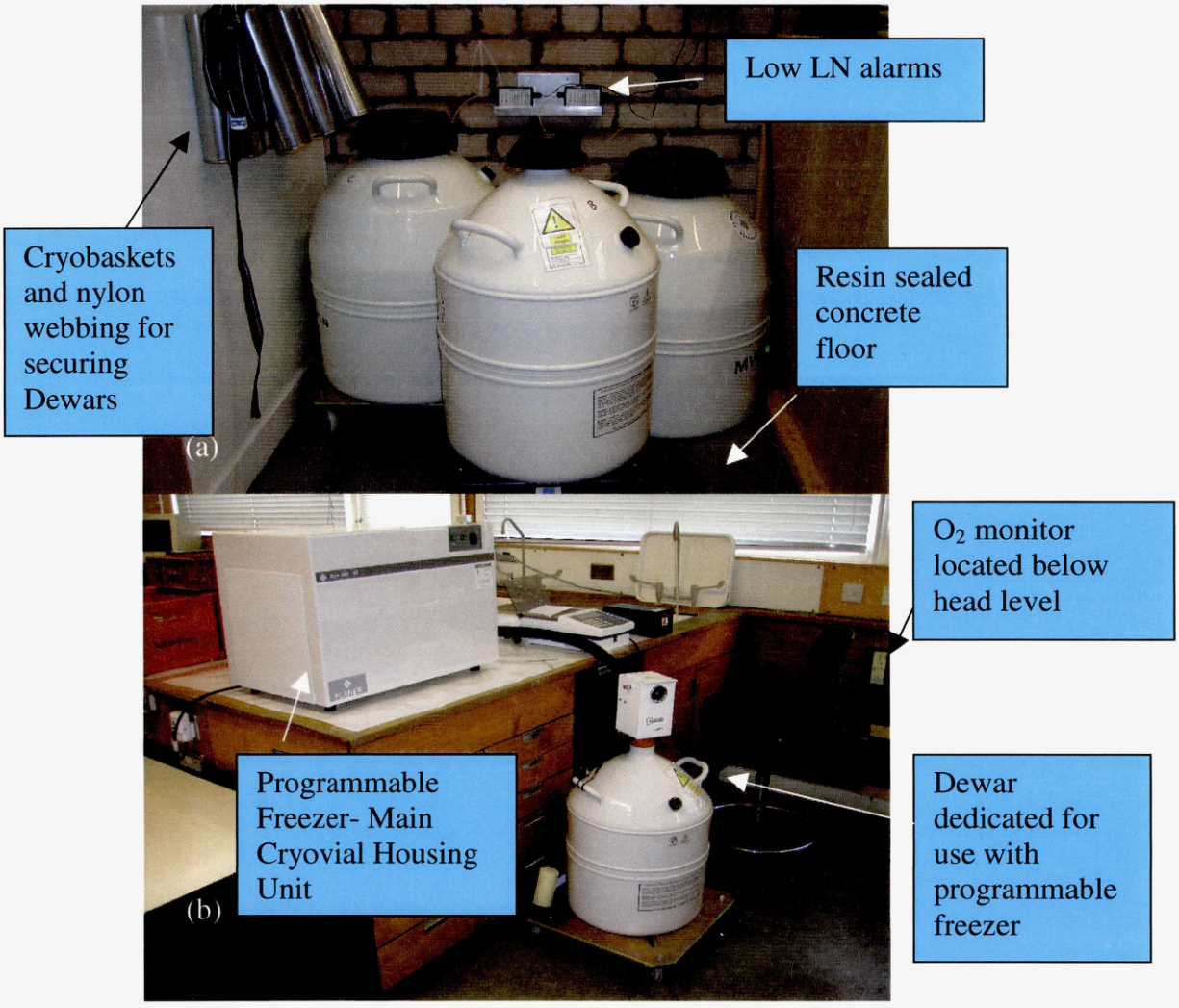


Figure 6.3 Cryopreservation features of (a) NRS LN storage Dewar area (b) NRS main *in vitro* laboratory, housing Planar programmable freezer.

6.2.3 Cryopreservation

6.2.3.1 Skills transfer

Two NRS research staff spent one day at UAD where they underwent training in ESM cryopreservation in accordance with the direction provided (Chapter 2). The two NRS staff then trained three BSc sandwich students who ran preliminary cryopreservation testing and implementation. In accordance with the original operating procedures recommended for ESM cryopreservation a critical points table was designed, so that staff could be questioned on each point (Table 6.1). Cryopreservation programs were developed and optimised for transfer from UAD, the cryo-research institute, to the NRS, for applied forestry research.

6.2.3.2 Cryopreservation protocols

The optimisation and final protocol production for these explants has been outlined in other chapters in this project. A controlled rate cooling protocol was optimised for ESM (Chapter 2) and an encapsulation-dehydration system for partially matured somatic embryos (Chapter 3). The cryopreservation of conifer ESM using a controlled rate cooling protocol has been proven successful with conifer suspension cultures (ES) (Cyr, 1999) and, will be the prototype protocol for use in a *P. sitchensis* genebank. It is anticipated that the technology transfer of encapsulation-dehydration procedures for somatic embryos will follow. It was recommended that NRS cryopreserve 10 replicate cryovials per cell culture line e.g. A5 (1) for each of Dewars.

Table 6.1 Critical factors were prepared following skills transfer discussion to assist with the first technology transfer evaluation.

Factor	Specific points
Personnel	- Basic laboratory skills proficiency
	- *In vitro* manipulation skills
	- Stringency to protocol procedures and thoroughness
	- Regular personnel or variable
Source ESM status	- Genotype and family characteristics
	- Time in culture
	- Subculture transfer interval
Step by step instructions and protocol interpretation	- Cryoprotectant solution preparation
	- Controlled Rate cooling protocol
	- Encapsulation-dehydration protocol
	- Controls for cryopreservation and LN handling
	- Re-warming and rehydration
	- Rinsing and plating
	- Post-treatment transfers
General facilities and sterility	- ESM culture facilities
	- Laminar-flow benches
	- Water bath
	- General laboratory facilities
Culture	- Growth medium
	- Growth regulators
	- Standard culture regime
Pre-growth and recovery	- Pre-culture (Medium preparation and application)
	- Pre-treatment
Cryogenic facilities	- Type of Dewar
	- Type of programmable freezer
	- Cryo-Vials, canes, boxes
	- Labeling and inventory system
	- LN nitrogen availability and top-up system

Viability testing

Associated viability tests were also established

(1) Fluorescein diacetate (Chapter 2) for ESM

(2) Tetrazoilum triphenyl chloride for ESM and somatic embryos (Chapter 3) (Steponkus and Lanphear, 1967)

Contamination

A test to detect the presence of systemic bacterial contamination was developed and it is recommended that the test is undertaken on a fast proliferating genotype of ESM, prior to cryopreservation and at least annually throughout cryo-storage. A test to determine the presence of mycoplasma or systemic infection was also developed at UAD. ESM was incubated in ½ strength liquid MS medium with 256mg/l of peptone and 88mg/l yeast extract at pH 6.7 for 1 week and then placed on standard solid MS medium under routine culture

conditions. Contaminated cultures were usually observed within 72hr of liquid incubation, as the clear media became cloudy and these cultures were discarded.

Marker assisted culture characters

(1) Photographs to compare parental colour and morphology (such as friability)
(2) Microscope viewing of ESM shows suspensor tail to embryogenic head ratio often indicative of cryotolerance and age.
(3) Fresh weight increase comparisons

6.2.4 Records, cryovial/cane identification and documentation

Cryo-canes will be accurately referenced at NRS through colour tab markings and individual cryovials through a stamped cell bank reference number. In technology transfer discussions it was suggested that since the cryocanes are soft aluminium they can easily be stamped using 4mm punches. Three characters one letter and two numbers gives the possibility of over 2,500 distinct combination possibilities.

The reference numbers will be recorded through a printed inventory and database. The database may be a shared Access/Excel file (as is shown in Table 6.1) or if there are multiple users in different locations then a secure online database such as the COBRA website www.cobra.ac.uk may be required. The COBRA database searches are based on strains of algae, but the principles equally apply to most cryopreservation systems and genotypes as in the *P. sitchensis* breeding program. NRS is in the process of implementing an online inventory system.

Table 6.2 Example of Inventory - Entry required per cryo-cane, staff member expected to check that no duplication of cryo-cane colour per housing unit.

E.g Working LN Dewar 1

Entry date/time Staff member/ID number	**Species/explant type/cell culture number**	**Number of cane holding unit in Dewar**	**Colour/code number of cryocane top**	**Number of cryovials on cryocane**	**Withdrawal date/time Staff member/ID number**
12/09/05 12:05 SG-15001	*Picea sitchensis* Embryogenic cell masses A5 (1)	5	Purple SG1	4	
12/09/05 12:05 SG-15001	*Picea sitchensis* Embryogenic cell masses A2 (1)	5	Red SG2	3	

Genotype
(Batch initiated)

6.3 Validation of protocols, equipment and training

After the development of suitable cryopreservation protocols at UAD, technology transfer took place over two years until post-LN success was reported in August 2005. (Fig. 6.4)

Figure 6.4 Chronology of cryogenic development technology transfer to NRS

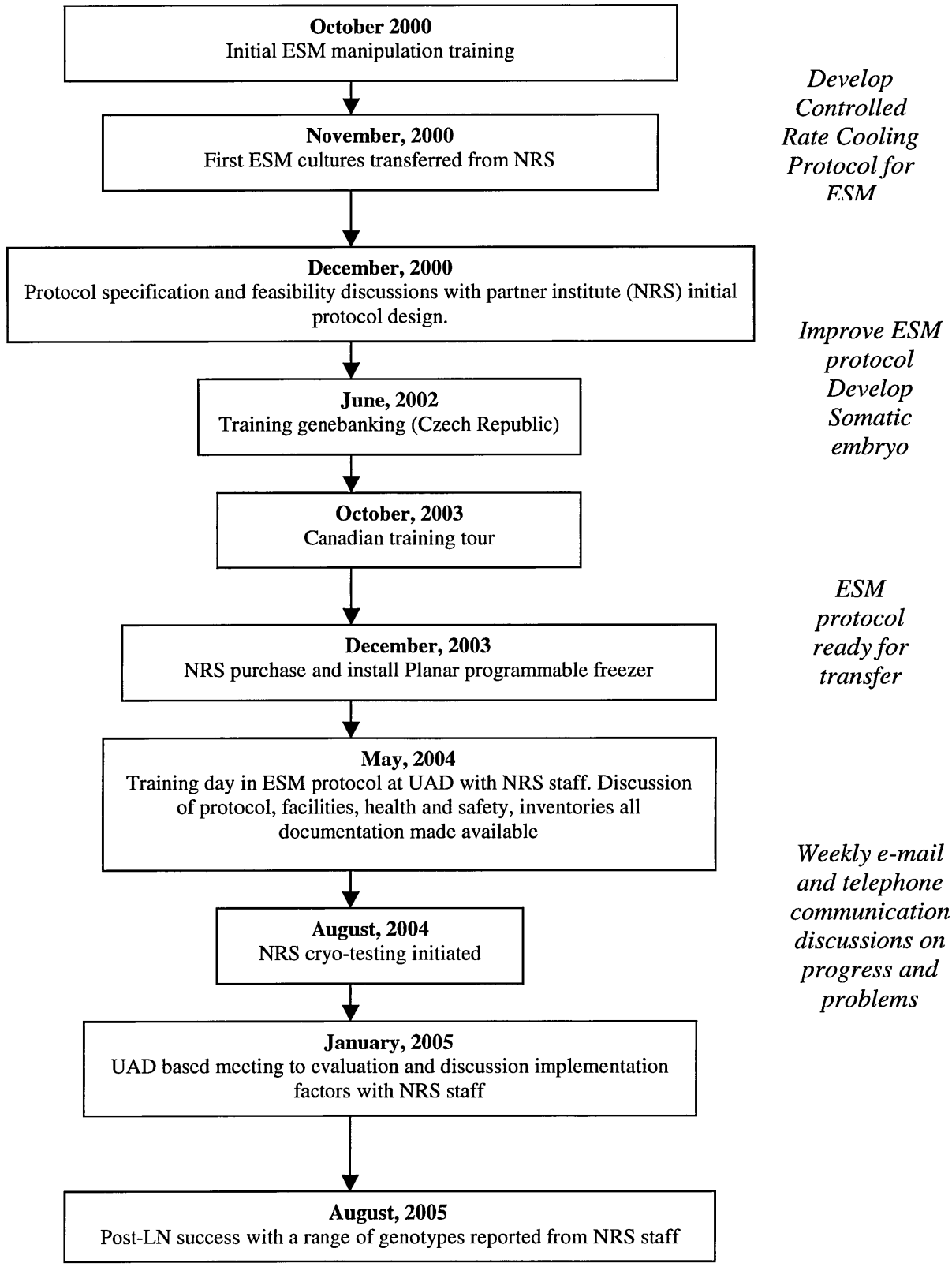


Table 6.3 Identification of problems, critical factors and corrective procedures following technology transfer

Details of problem	**Possible Interpretation**	**Identification of Critical Factor following discussion**	**Corrective procedure following discussion**	**Corrective action response**
Controlled Rate freezer profiles show ice nucleation at a higher temperature (-8°C) at NRS than UAD (-10 to -12°C)	(1) Cryoprotectant not prepared correctly? (2) Too much ESM in cryovial diluting cryoprotectant? Protocol specification 'fill cryovial ¼ to ½ with ESM' ambiguous terminology.	Interpretation of Protocol	(1) Attempt to stabilise correct ratio using printout as a guide and insure staff member can reproduce and demonstrate OR (2) Weigh ESM prior to cryopreservation in flow bench. (Less favourable because adds a another step, and increases risk of contamination and Cell damage.)	NRS staff tested small pieces of ESM in an excess of cryoprotectant during the pre-treatment and then transferred them to cryovials for cooling in PRF*. Post-LN survival of ESM cultures was 8%.
No-post cryopreservation recovery	Any one of identified points- discussion required across all.	NRS cultures tested not same as UAD cultures.	(1) Genotype or (2) Culture age	Cryopreserve cultures within 3 months of initiation.
Staff unsure when to remove cryovials from water-bath during re-warming and leaving cryovials in water bath for up to 5min.	Protocol indicates that cryovials should be removed 'after approximately 2 min or when ice plug eliminated.'	Interpretation of protocol	(1) Protocol requires clarification – not to maintain cryovials in water bath for longer than 120 sec. (2) Testing on control cultures to determine ESM recovery following immersion in 40°C water-bath for 60–300 sec	Unfrozen ESM was tested in the water bath at 40°C for 40 to 300 sec and all survived with no indication of damage.

Detail of problem	Possible Interpretation	Identification of Critical Factor following discussion	Corrective procedure following discussion	Corrective action response
Cryoprotectant and ESM are stirred together with mini-stirrer in cryovial	The action of the stirrer may be damaging ESM cells and increases the risk of contamination from handler	Protocol interpretation	Adhere to protocol gently flick cryovials or use a vortex to mix cryoprotectant with ESM.	Implemented

PRF*= Programmable Rate Freezer

Italic indicates the problem interpretation identified as the most accurate explanation through discussion with NRS staff.

The meeting with NRS staff (Dr. Allan John), January 2005, in Dundee, proved to be the most effective time in identifying problems and offering corrective procedures and the problems listed in Table 6.3 were derived from this structured meeting. Dialogue in person with diagrams and printouts from the programmable freezer apparatus indicated that a major problem was in determining the correct ESM to cryoprotectant ratio without adding a weighing step where desiccation and contamination might further complicate recovery. Once staff were aware of the problem a series of tests were undertaken so that staff could recognise the optimal volume of ESM that should be placed in each cryovial. Other factors relating to ESM culture age, re-warming protocol and ESM manipulations were discussed and resolved in the same format.

6.4 Summary of progress

This chapter has identified technical, operational and practical issues associated with the development and transfer of cryopreservation protocols for *P. sitchensis* ESM germplasm between partner institutes. A protocol best fitted to the operating and facility based-specifications (such as the purchase of a programmable freezer) of the recipient partner institute (NRS) was selected for technology transfer. Controlled rate cooling protocols were already reported for similar, embryogenic suspension (ES) conifer germplasm and provided a starting place for experimental development for *P. sitchensis* ESM (Chapter 2).

During the transfer of technology critical point factors were most easily identified firstly through person-to-person meetings and secondly through e-mail and telephone discussions. Critical factors included protocol clarification, *in vitro* culture age and adhesion to original operating guidelines and required corrective actions from the trainee partner institute. Modifications to the operating procedures at NRS resulted in successful post-LN recovery of ESM germplasm. Critical factors established in this study are similar to those reported by other cryogenic practitioners (Reed, *et al.*, 2001, Reed, *et al.*, 2004, Stacey, 2004) who have established the foundation for cryopreservation technology transfer.

6.5 Improvements to technology transfer

Technology transfer may have been facilitated through a longer UAD–based cryopreservation protocol training session (2-3 days) and through additional training (1 day) on–site at NRS once equipment was installed and operational. Technology transfer may have been facilitated through the provision of a questionnaire drafted by UAD training staff to NRS trainee staff one month after the delivery of the ESM Operating Procedures to establish baseline comprehension and application. This was determined to be extremely effective in technology transfer across international plant conservation laboratories (Reed, *et al.*, 2004).

6.6 Conclusions

Cryopreservation techniques and protocols have been successfully transferred from UAD to the partner institute NRS and cryogenic facilities implemented. Preliminary cryopreservation testing shows that ESM cultures are showing post-LN recovery. A series of recommendations generated from cryopreservation development at UAD and study tours undertaken will further assist in post-LN recovery optimisation at NRS. A key recommendation is that ESM cultures are cryopreserved <3 months following initiation. Implementation of the somatic embryo (see Chapter 3) cryopreservation programme and NRS operating systems will proceed outside the time frame of the study.

Chapter 7 GENERAL DISCUSSION

7.1 Introduction

The aim of this research project was to develop cryopreservation protocols and establish a prototype genebank for *P. sitchensis* germplasm. Three *in vitro* explant types (embryogenic suspensor masses, somatic embryos and shoot-tip apices) were targeted for cryo-storage with *in vitro* physiology characterisation and growth monitoring used to assist in cryopreservation protocol development. This overview highlights key research findings from both fundamental and appled research, and evaluates the project outcomes in terms of forest genetics resources conservation.

7.1.1 Key findings

1. *P. sitchensis* germplasm cryopreservation methodology has been developed and the UK's first forestry cryopreservation genebank established.

2. Cryopreservation protocols have been successfully developed for ESM and somatic embryos of Sitka spruce for the first time and steps progressed in overcoming *in vitro* and cryogenic shoot-tip apex recalcitrance in this species. Also for the first time, the re-initiation of ESM and NEM from cryopreserved somatic embryos was achieved as a possible mass multiplication pathway for germplasm production.

3. Major steps were progressed for one of the most recalcitrant *in vitro* explants to date, shoot-tip apices of conifers, with critical pre-treatment steps established. An improved understanding of conifer shoot-tip recalcitrance has been achieved by relating the ecophysiology of conifers *in situ* to performance *in vitro*.

4. The major physiological impacts of microenvironmental differences, especially prolific in light-impacted heterotrophic culture, are for the first time reported in *in vitro* growth facilities set to the same general parameters. A biochemical tool kit to measure markers of oxidative stress, stress hormone production and DNA methylation has proven an effective research approach.

5. Importantly in this study cryogenic and non-cryogenic critical factors have been assessed independently, by applying thermal analysis (differential scanning calorimetry) and general trends in vitrification and explant water relations compared.

7.1.2 Discussion framework

There were two key aims at the start of this project: (1) a fundamental objective, to use physical, physiological and biochemical studies to assist the development of cryopreservation protocols for Sitka spruce, and (2) an applied objective, to design cryopreservation protocols for different explant types within the feasible operational parameters of the end-user, the recipient institute and to transfer recommendations for routine cryobanking.

The fundamental achievements and contributions to scientific knowledge from the thesis are structured through the two key aspects of cryopreservation. Firstly, non-cryogenic factors (genotype, *in vitro* age, developmental stage) are considered in relation to specific explant physiology. Hence each explant is considered distinctly but in logical progression of anatomical complexity (7.2 to 7.4). Secondly, the cryogenic and biophysical factors (water status and interplay of pre-treatment and cryoprotectants relating to ice formation or amorphous glass formation) determined through thermal analysis using the DSC, (7.5).

Discussion of the applications of the fundamental findings of this study, in terms of benefits to this project and industry at large, specifically UK forestry, will be structured in two ways. Firstly, in terms of how each specific explant cryopreservation system (7.2 to 7.4) can be utilized and implemented for this project with recommendations for future development, and assist with broader industry applications. Secondly, the whole process of technology transfer, protocol selection and the issues raised from this study will be discussed (7.6). Recommendations in regards to the implementation of this cryo-facility, with more far-reaching implications for forest genetic resource cropreservation development and cryobank establishment will be outlined.

7.2 Embryogenic suspensor masses

Fundamental

Cryopreservation testing was initiated with ESM because (a) it was the anatomically the simplest explant of the three in this study, and (b) a successful protocol was already developed for *P. sitchensis* Embryogenic Suspension (ES) (Find, *et al.*, 1993) based on controlled rate cooling methodology. This cryopreservation protocol was successfully modified for the more heterogeneous ESM and fresh weight proliferation rates of ESM equivalent to untreated controls were regained within the second subculture (at 84 days post-cryopreservation) in four of five genotypes tested. In long-term assessments, (2-13 months

post-cryopreservation) ESM culture proliferation rates were comparable or greater than the control cultures in all genotypes. Further assessments demonstrated that these cultures were able to produce somatic embryos following cryopreservation. The protocol was applied to 25 genotypes, 19 survived, and the protocol was validated over 3 years, the first time that such an extensive study has been reported for *P. sitchensis in vitro* cultures.

The extensiveness of the study highlighted the two critical non-cryogenic factors of post-LN recovery of ESM as the genotype and *in vitro* age. This study indicated that the embryogenic capacity of the genotype influenced ESM recovery not the genotype *per se*. Interestingly, there were often more differences in post-LN ESM recovery between genotypes of the same family, even though they shared half the genetic compilation to each other, than unrelated genotypes of different families. This study corresponds well with that conducted on thirty unrelated genotype families of *P. glauca* where it was also concluded that there were no apparent family differences in post-LN effects (Park, *et al.*, 1998).

Cryopreservation development was assisted by vital staining fluorescein diacetate (FDA) and importantly re-affirmed that the distribution of cryotolerant cell clusters was heterogeneous within the ESM matrix. However, its sole application as an indicator of survival is not recommended since survival in the sample cluster may not represent survival in the whole test cryovial.

Post-LN recovery from ESM indicated that there were more vigorous genotypes (A5 and B1) and less vigorous genotypes (D3 and E3) on a fresh weight criteria (Table 2.7). This order was generally adhered to following the sorbitol pre-treatment and cryoprotection application selection forces. In some cases the post-sorbitol treated ESM growth rate surpassed the control. It is theorised that sorbitol may have exerted osmotic selection allowing younger, more vigorous and meristematic (and possibly embryogenic) cells to proliferate more rapidly in the absence of older cells. In the same way cryopreservation, may have applied a selective pressure for cells of suitable size and morphogenic capability to tolerate the cryogenic processes.

In the same way, culture vigour and embryogenic capacity were essential for somatic embryo production, both in terms of number and quality. Genotype A5 showed the greatest culture diameter increase and produced size 4 embryos, the level of development reported as optimal for successful transfer and germination (Gupta and Grob, 1995). Culture diameter may be important for the production of healthy embryos as it has been observed in newly maturing

embryos that development irregularities can occur when embryos come in to contact with each other (Svobodova, *et al.*, 1999).

This study also ascertained that the initial embryogenic capacity of a genotype is not constant and may decrease over time. Two genotypes in this study showed a complete loss of embryogenic capacity after 3 years in *in vitro* culture (36 subcultures). The cause of the embryogenic loss is unknown and was not investigated in this study, but other studies such as those undertaken on rice cells have determined a higher antioxidant status with non-embryogenic cells than in embryogenic cells, possibly indicating greater free-radical stress (Benson, *et al.*, 1992). *In vitro* aged cultures may generate larger stressed cells that are likely to have lost their meristematic characteristics making them more difficult to cryopreserve due to their water status.

Cryopreservation trials illustrated that the most suitable protocols for ESM were those that circumvented laminar-air flow desiccation methodology. Desiccation trials of encapsulated ESM showed that more than 2hr of laminar air-flow desiccation resulted in the complete lack of ESM recovery. It was hypothesised that the moisture content would still be very high in these beads and this was confirmed by MC trials and DSC analysis. Indeed thermal analysis demonstrated that a minimum of 4hr laminar air-flow desiccation was required to ensure no ice was formed during cooling or upon re-warming (Tables 5.10 and 5.11) and so the limited post-LN recovery (20% in 1 of 5 genotypes tested, Fig. 2.9, Chapter 2). This was interpreted to be due in part to a sub-optimal desiccation phase resulting in the formation of lethal intracellular ice.

Applied

The protocol provided the first method in which *P. sitchensis* germplasm can be cryopreserved, stored in LN and recovered to produce elite trees of known genetic origins for commercial foresters. This protocol can now be integrated into the UK Forestry Commission's Northern Research Station *P. sitchensis* breeding program and will be applied to 50 families over the next three years, each family containing 10 genotypes. This will be the first time cryopreservation has been incorporated into tree improvement programs in the UK and will be the first UK Forestry Commission cryobank and it will serve as a prototype for other conifer or non-conifer tree improvement programs.

Following this study and recommendations from other conifer culture specialists who regularly achieve 99% post-LN recovery in *Picea* sp (Dr. Yill Sung Park, per. comm.) it is

recommended that ESM should be cryopreserved within 6 months of initiation. This time-scale may also have implications for genetic stability.

This study also reports for the first time on the post-LN timescale, an aspect often overlooked by researchers developing protocols for large–scale industry. It may not be feasible to mature small batches of culture immediately after recovery from LN and the genebanker may need to bulk up ESM for industry demands. In this study, it was observed that ESM maturation capabilities were not affected by post-LN culture for up to 1 year.

7.3 Somatic embryos

Fundamental

Somatic embryos are at the next stage of complexity from ESM and have been the subject of much less research in conifer cryopreservation. Pre-treatment parameters for cryogenic optimisation of ESM assisted in somatic embryo protocol development, but cryopreservation strategy was adjusted for the physiology of the explant. Encapsulation–dehydration methodology was tested but it was also anticipated that in parallel with seed–based zygotic embryo development, somatic embryos may show greater desiccation resistance. Encapsulation-dehydration methodology (incorporating a 4hr laminar air-flow desiccation) was successfully applied to the somatic embryos and up to 50% post-LN TTC viability achieved (Fig. 3.11). Developments in PVS2– vitrification of naked somatic embryos were also achieved. In combination with cryopreservation success this study reports for the first time the re-initiation of embryogenic suspensor masses (ESM) (up to 20% post-LN, Fig 3.12) and non-embryogenic masses (NEM) from Sitka spruce somatic embryos. This methodology could make somatic embryo cryopreservation a very important and viable pathway for germplasm storage and rapid generation.

In common with ESM cryopreservation, both the genotype and the *in vitro* age of the donor ESM culture (somatic embryo source) affected the success of post-LN recovery and ESM re-initiation. However, the additional complexity of the explant offered another variable: the developmental size of the somatic embryo.

The framework of the experiment was influenced by which ESM source culture genotypes could (a) produce somatic embryos and (b) in sufficient numbers for replicated testing. This meant that only four genotypes were utilised, two (A1 and A5) from the same family; the A family has already been noted as showing particularly strong growth vigour in section 7.2. Although genotype A1 is derived from the same family as A5, no ESM re-initiation was observed regardless of the treatment. As with the ESM cultures a basis to the differences of

embryogenic trends between genotypes was not determined in this thesis, but other studies akin to this one show comparable responses. Recovery response variability between clones within families of *P. glauca* somatic embryos (Park, *et al.*, 1994) was attributed to variability in culture quality rather than genetics. The embryogenic tissue re-initiated from treated somatic embryos was comparable to parent embryogenic tissue of that genotype.

There was a 6-month difference in the age of source embryogenic cultures (6 subcultures). The percentage of ESM re-initiation in December tested control embryos (A5 and C5) was half that of equivalent July tested embryos, and so it was concomitantly realised that somatic embryo ESM re-initiation capability was also associated with the *in vitro* ageing of the source cultures.

Results from relative moisture trials (Fig. 3.8) of encapsulated size 2 and 3 somatic embryos undertaken during laminar air-flow desiccation do not suggest that the larger encapsulated embryos contain more water. Size 3 embryos showed post-cryopreservation ESM re-initiation, but immature size 2 embryos showed superior TTC viability, possibly because they are anatomically more meristematic and less vacuolated.

The critical component for this protocol to be readily accepted into forestry industries is the re-initiation of ESM, a viable germplasm clonal mass that can be rapidly propagated, matured and deployed. At this stage, the validity of ESM re-initiation as a useful route for *in vitro* germplasm generation and morphogenic capability, will require further testing. However the NEM proliferation was recorded and not disregarded, because there are regular developments in *in vitro* culture physiology and it is anticipated that the switch between callus and embryogenic callus will be better understood in the future. An investigation of the effects of plant growth regulators, carbon sources and iron on secondary somatic embryogenesis in transgenic cherry rootstock suggested that 1mg thidiazuron (TDZ) with 2% (w/v) sucrose reverted morphogenetic callus to non-morphogenetic callus (Gutierrez and Rugini, 2004). Genetic stability will need to be considered carefully for this route of plantlet production since dedifferentiation can induce more genetic changes than regeneration through a differentiated route and may be associated with somaclonal variation problems (Harding, *et al.*, 1996).

Applied

The successful cryopreservation of *P. sitchensis* somatic embryos allows new possibilities and applications for the genebanker. Although at present the encapsulation of conifer somatic embryos as a means of cryopreservation is in the preliminary stages of investigation, somatic

embryo cryopreservation could provide a cost-effective storage strategy that may be automated with a bioreactor, with no need for a programmable freezer.

Somatic embryos may be used to create synthetic seed where they might undergo *in vitro* manipulations and cryogenic processes in a Na-alginate shell (Gray, *et al.*, 1995, Gray and Purohit, 1991). The choice of synthetic seed direct germination or multiplication through embryogenic tissue re-initiation would meet market requirements, which may be the production hundreds of thousands of clonal emblings within months of recovery from storage.

7.4 Shoot-tip apices

Fundamental

The most complex *in vitro* explant, in this study, is the shoot-tip apex. The study of which, required a holistic and novel approach to make progress in cryopreservation protocol development. It was clear that a fundamental understanding of the explant, and characterisation of its *in vitro* physiology was necessary before cryopreservation protocols could be applied.

To understand *in vitro* and cryogenic recalcitrance in of *P. sitchensis* shoot-tip cultures, it is important to consider the adaptations of the species *in situ* and the processes applied to produce *in vitro* cultures. This study therefore incorporated a review of the species ecophysiology, breeding and *in vitro* genesis of shoot-tip apices. Thus indicating how endogenous genetic control is maintained through *in vitro* culture. It was clear that cryopreservation development in this study was influenced by: (1) the endogenous genetic pattern persisting *in vitro* and manifested as shoot growth flushes and dormancy and senescence, and (2) sensitivity to microenvironment change.

Physiological indicators for inter-locational and genotypic differences in growth were also observed. After 6 months of culture the growth rate of shoots from the same genotype (AC) after 42 days at NRS was observed to be nearly double that of shoots at UAD (Fig. 4.6). UAD cultures also exhibited other physiological differences including extended internodes, yellowing and bunching of needles (Fig. 4.5). In the case of this explant, it was determined, that an additional *in vitro* characterisation with a quantitative basis was required so that when cryopreservation was applied cryogenic and non-cryogenic factors could be clearly differentiated.

Biochemical methods incorporating oxidative stress, free radical attack, stress hormones and DNA methylation were applied. The primary aims of these studies were to: (a) develop a set of diagnostic markers that can be utilised as a stress indicator at any stage of *in vitro* culture or cryogenic manipulation and (b) apply them in this study to investigate physiological degradation seemingly associated with genotype and microenvironment changes. However, this study also contributes to the fundamental understanding of epigenetics and *in vitro* plant culture.

Short-term post-cryopreservation shoot-tip recovery was initially achieved for *P. sitchensis* shoot-tips using encapsulation-dehydration methodology. Important non-cryogenic criteria for a successful cryopreservation protocol in shoot-tip apices were identified as pre-treatment steps of cold hardening pre-culture with sucrose, proline or ABA and a short post-dissection DMSO treatment. A longer cold hardening phase in theory is the perfect natural mechanism for cryopreservation, however it was difficult to control and unreliable as a biotechnology application

Applied

Although fundamental knowledge has been contributed and progress achieved, further work is required to make use of shoot-tip cultures as a useful germplasm storage and propagation explant. It seems unlikely that these shoot-tip cryopreservation methods will be progressed for *P. sitchensis* by the UK Forestry Commission because of *in vitro* recalcitrance problems and the time and the high skill input to low offspring output as compared to ESM and somatic embryo methodology. However, these studies will be of considerable interest to other areas of plant conservation. There are interests in developing *in vitro* shoot-tip cryopreservation methodology for recalcitrant tree species where: (a) *in situ* germplasm resources are threatened or endangered (b) genetic conservation at a population level is required (shoot-tip culture shows highest genetic preservation), and/or (c) no other explant or germplasm source can be reliably obtained or cryopreserved.

7.5 Cryogenic comparisons using the DSC

Cryogenic factors and vitrification stability were investigated through thermal analysis using a Perkin-Elmer differential scanning calorimeter, in three *P. sitchensis* explants. Optimised pre-treatment regimes determined from post-LN recovery and viability, developed for explants in Chapters 2-4, corresponded well with thermal stability and the elimination of damaging ice events and in all cases excluding the encapsulation-dehydration of ESM, there was no physical cryogenic basis for post–LN recovery failure.

In all cases, DSC information assisted in pinpointing water contents in relation to osmotica pre-treatments, laminar air-flow desiccation requirements and vitrification treatment exposure obtained. In all cases water decreased progressively with dehydration and desiccation treatments.

Under optimum pre-treatment regimes, osmotically active water was completely removed but the relationship of osmotically inactive water to survival and biological interpretation is not yet fully understood. The more complex cell composition of the shoot-tip apices and consequent differential penetration and dehydration modes of sugar and cryoprotectant treatment may explain why shoot-tips were so recalcitrant to cryopreservation. *In vitro* cold-hardening may offer a useful pre-treatment method, as the complex intracellular solutes accumulated to protect against ice and affect osmotically inactive water content.

Many thermograms were complex, especially those with PVS2 treatment. Thermal behaviour is influenced by the water content, pre-treatment history prior to glass formation, and the complexity and heterogeneity of glassy matrixes and their cellular components. Ice nucleation was eliminated in all cryoprotective strategies of optimised pre-treatments to produce stable thermal profiles. The profiles produced correlate with other species where post-cryopreservation survival has occurred. This suggests that these protocols are thermodynamically stable and that other factors are responsible for explant survival.

Na-alginate encapsulated explants showed similar thermal profiles. Following encapsulation-dehydration, blank beads and encapsulated shoot-tip apices and somatic embryos generally behaved in a similar fashion throughout progressive desiccation. Somatic embryos showed greater osmotically inactive (OI) water than shoot-tip apices, and further work must be undertaken to determine, if this difference is due to variability in lipid content due to explant physiology and/or pre-treatment stress (cold-hardening).

In encapsulation-dehydration treatments bead size was an important factor, although sucrose dehydration assured some degree of homogeneity. Further controls might be required to assure precise rates of desiccation. PVS2 treated beads showed less variability in total water and OI water between replicates and where bead size was not so critical. Naked somatic embryos required a simpler PVS2 treatment compared to encapsulated explants. These studies focus attention to control relative humidity, bead size and transfer timing between operations as water status is very sensitive to these critical factors.

7.6 Implementation of cryopreservation programme for UK forestry

Following the development of optimised protocols, ESM cryostorage methods were transferred to the partner institute, NRS. ESM was selected because in the cryopreservation trials it showed the highest and the most consistent post-LN recovery. Technical, operational and practical issues associated with the development and transfer of cryopreservation protocols for *P. sitchensis* ESM germplasm, between partner institutes were discussed (Chapter 6). A protocol best fitted to the operating and facility based-specifications (such as the purchase of a programmable freezer) of the recipient partner institute (NRS) was selected for technology transfer. Low technology based cryopreservation protocols such as Mr. Frosty™ for ESM and encapsulation-dehydration for somatic embryos were also available for future implementation for NRS.

During the transfer of technology critical point factors were most easily identified firstly through person-to-person meetings and secondly through e-mail and telephone discussions. Critical factors included protocol clarification, *in vitro* culture age and adherence to original operating guidelines and required corrective actions from the trainee partner institute. Modifications to the operating procedures at NRS resulted in successful post-LN recovery of ESM germplasm. Critical factors established in this study are similar to those reported by other cryogenic practitioners (Reed, *et al.*, 2001, Reed, *et al.*, 2004, Stacey, 2004) who have established the foundation for cryopreservation technology transfer. Post-LN survival of ESM cultures was improved following protocol clarification and identification of critical point factors.

7.7 Conclusions

1. A cryopreservation protocol, using a programmable freezer (Planar), was successfully developed and is reported for the first time for *P. sitchensis* embryogenic suspensor masses (ESM). Post-LN survival rates of up to 100% were observed in several genotypes (Fig. 2.14).

2. Encapsulation-dehydration was successfully utilised to cryopreserve *P. sitchensis* mature somatic embryos reported for the first time. Recovered embryos were able to re-initiate into dedifferentiated NEM (up to 100%) and ESM (up to 20% post –LN) as a source of material to mass multiply cryopreserved clonal offspring.

3. This study has affirmed that there are difficulties in prolonged explant *in vitro* culture and *in vitro* culture ageing associated with explant developmental morphology, embryogenic capacity and in some cases cryo-tolerance. Based on the evidence attained in this study and on recommendation from established woody conifer cryobanks in other countries, ESM and embryos need to be cryopreserved as soon as possible and at the latest within 3 months of culture initiation.

4. Short-term post LN recovery was achieved following encapsulation – dehydration of *P. sitchensis in vitro* shoot-tip apices. An essential component of the protocol was to utilise cold treatment in the pre-culture preparation.

5. Fundamental studies of *in vitro* conifer shoot-tips reaffirm culture difficulties are due to (a) *in vivo* generated growth problems manifested because of extreme ecophysiological adaptations of the donor plant often resulting in dormancy and growth flush patterns, and (b) the particular sensitivity that shoot-tips have over other germplasm explants (ESM and somatic embryos) due to micro-environmental changes, presumably pertaining to light-impacted regulation.

6. For the first time, a set of diagnostic biochemical tools (incorporating oxidative stress detection and DNA methylation) was applied to *P. sitchensis* shoot-tips, to better characterise the shoot-tip cultures and assist in ameliorating environmental stress and storage recalcitrance.

7. Thermal analysis (using the differential scanning calorimeter) was applied to all three *P. sitchensis in vitro* explants for the first time. DSC analysis indicated that thermal stability without ice events correlated well with survival data, and assisted in pinpointing water relations following osmotica pre-treatments, laminar air-flow desiccation requirements and vitrification treatment exposure obtained.

8. DMSO in varying applications and concentration, appeared to show a multi-faceted role throughout this study as pre-treatment antioxidant free radical scavenger (following shoot-tip apex dissection); as a colligative cryoprotectant before and during cooling (penetrating cell walls to assist

entry of other cryoprotectants, supporting cell structure, preferential exclusion protecting proteins, and influencing unfrozen water content); and in rapid recovery of dedifferentiated ESM.

7.8 Scope for future work

7.8.1 Fundamental research

1. The absolute priority for the development of the storage protocol is to monitor the developmental progress of the cryopreserved emblings to fully mature trees to ensure that they have comparable *in situ* development and genetic stability, as compared to non-cryopreserved regenerants.

2. Further work is required to understand NEM and ESM physiology and biochemistry to determine if they are genetically stable.

3. The relationship between NEM and ESM requires study, and investigation to determine if it is possible to switch from one state to the other.

4. Fundamental studies and comparisons in the *in vitro* culture of shoot-tip and ecophysiology of *in vivo* shoots (whole plant physiology) of conifer species and other species exhibiting acute seasonal physiological adaptation should be considered in a framework developing optimal *in vitro* culture parameters.

5. *In vitro* cold acclimation studies in woody plant species should examine profiling sugars and amino acids as possible markers for physiological adaptations to *in vitro* stress and cryoinjury.

7.8.2 Applied research for conifer clonal forestry programmes

1. Continued optimisation of other pre-treatments for explants, such as carbohydrates (sugars–sucrose, glucose, maltose and sugar alcohols-mannitol) and cold acclimation, for effectiveness and economic value.

2. Encapsulation-vitrification methodology has proven to be very effective with previously recalcitrant woody plant shoot-tip cultures, but no post-LN success was achieved in this study. *Picea sitchensis* shoot-tip cultures that have been cultured for

a shorter time (less than 6 months) may respond better to the optimised pre-culture regime (outlined in this study) and encapsulation-vitrification with an additional component of polyvinylpyrrolidone (PVPP) to counteract phenolic oxidation if phenolics were detected.

3. Encapsulation-based methodologies successfully applied to somatic embryos may be more feasible, even through direct germination without an ESM or NEM phase, if they were compliant to automation and germination as, 'synthetic seed.'

4. Future research efforts would need to be focused on developing technologies prior to cryopreservation; including ESM culture, somatic embryo selection, osmotic pre-treatment, Na-alginate encapsulation, osmotic dehydration and desiccation; and following cryopreservation through recovery and maturation and germination as a single procedure.

Final Statement

This study has produced a set of protocols and recommendations that have assisted in the development of a prototype, pilot genebank for UK forestry cryopreservation, developed for *P. sitchensis* germplasm. The study however has far broader implications for transfer of cryopreservation protocols to other UK forestry breeding programmes and for forestry genetic conservation purposes. This study has shown that the cryogenic factors controlling cryopreservation success or failure can be controlled through optimising cryogenic factors based on a fundamental understanding of cryobiological principles. Such principles rely on water relations, ice physics and are controlled through water removal and cryoprotectant-water interplay. Key principles are rapidly being established in cryopreservation (Benson, *et al.*, 2005 In press) and generic similiarities are observed even across different explants and species. Thus, during controlled rate freezing or vitrification the same basic rules apply.
However, this study has highlighted the difficulties that still remain in *in vitro* culture and *in vitro* manipulations, especially with the more developed and complicated tissues, often through a fundamental lack of knowledge of the whole plant or organism physiology. This study re-emphasizes that the longer biological explants are maintained in an artificially stressful environment, often through artificially regulated chemical signals, the more risk there is of an imbalance resulting in physiologically recalcitrant and possibly epigenetic inherited change in clonal offspring. This is why *in vitro* culture should be considered as a temporary preparation stage for cryopreservation or bulk generation as opposed to storage *per se*. Therfore supporting the general view that cryopreservation is the major option for the long-term storage of *in vitro* plant germplasm.

Appendix

Glossary of specialist terms

Forestry related:
Clonal Forestry: Production of uniform trees of known genotype that shown proven and tested traits desirable for commercial forestry.
Traits: Desirable characteristics e.g. height, volume, form.
Heritability: The degree to which traits are inherited.
Plus trees: Trees showing superior traits.
Progeny: The offspring produced from trees showing elite traits.
Grafting: The union of a root system (understock) with a shoot system (scion) in such a manner that they subsequently grow and develop as one composite (compound) plant.
Half- siblings: Where pollination is not controlled and only the genetic inheritance from the mother (maternal) parent is known.
Full siblings: Where pollination has been controlled and both maternal and paternal inheritance are known.
Seed Orchard: A planting used in forestry to maintain seed sources either as seedling populations of selected seed families or of a grafted collection of clones.

In vitro physiology
Adventitious: Development of organs (roots, buds, shoots) or embryos from unusual points of origin, (and not from an original meristem), including callus.
Apical dominance: The phenomenon where the terminal bud of a shoot suppresses the outgrowth of the axillary buds.
Apical Meristem: A group of meristematic cells at the apex of a shoot (or root) by which cell division produce the primary tissues.
Autotrophic :Not requiring added organic substances
Axillary: Originating in the axils of the leaves
Callus: Actively dividing non-organised tissues of undifferentiated and differentiated cells often developing from injury or in tissue culture.
Cytokinins: Plant hormones (natural or synthetic), which induce cell division and often adventitious buds (shoots) and in most cases inhibit adventitious root formation; cytokinins decrease apical dominance.
Differentiation: The development of cells or tissue with a specific function or regeneration of organs or organ-like structures.
Embryogenesis: Process by which an embryo develops from a fertilized egg cell or asexually from a group of cells.
Embryogenic suspensor masses: Embryogenic de-differentiated/partially differentiated cultures, consisting of an heterogenous array of proembryo stages usually with embryogenic head (meristemoid) and suspensor tail formation.
Embryogenic suspension cultures: Liquid embryogenic suspension cultures maintained on a rotary shaker to agitate the masses and prevent the formation of large embryogenic clumps.
Epigenetic variation: Reversible variation; often resultant of changed gene expression, current studies are undecided about heritability, unproven studies show it may be heritable (K. Harding pers comm.).
Gibberelins: Group of plant hormones which induce cell elongation and cell division.
Habituation: The phenomena by which cells grow without addition of hormones following initial dependence.
Hyperhydricity: Translucent, water-soaked succulent appearance that can result in cultures that deteriorate and fail to proliferate.
Initiation: The start of the formation of a structure or an organ
Intercalary: Between two adjacent buds on a stem, within an internode
Juvenile phase: The period in the life of a plant during which no flowering can be induced. Within the juvenile phase the plant exhibits special features (morphology, physiology) that are different from the adult phase.
Meristem: Collection of dividing cells in the tip of a root, shoot, in the intercalary cambium of buds, leaves and flowers.
Micropropagation: Vegetative propagation of plants *in vitro*
Morphogenesis: The origin of form and differentiation of associated internal structural features.
Nucellar embryo: Embryo developed vegetatively from somatic tissue surrounding the embryo sac, rather than by fertilisation of the egg cell.
Organogenesis : Formation of a part of a plant with a specific function
Osmotic potential: Potential brought about by dissolving a substance in water; 1 mole of glucose per litre of water generates an osmotic potential of 22.4 atmospheres.
Polyembryony: When two or more embryos are formed after fertilisation
Plant growth regulators (PGRs): Synthetic or natural chemicals that show hormonal effects
Protocorn: Tuberous-like structure formed when orchid seeds germinate
Protoplast: Plant cell without a cell wall, produced by enzymatic degeneration
Rejuvenation: Reversal from adult to juvenile phase of development.
Shoot-tip: Apical (terminal) or lateral shoot meristem with a few leaf/needle primordia and leaves/needles.
Synthetic seed: Somatic embryos enclosed in an artificial seed coat. This may be a way of sowing somatic embryos for mass production.

Culture medium types
MS medium: Murashige and Skoog (1962) media selected for Sitka shoot-tip culture, no PGRs added.
SEMM: Somatic Embryogenic Maintenance Medium (John, *et al.*, 1995) developed for Sitka spruce embryogenic suspensor mass cultures [PGRs 1.105mg/l 2,4-dichlorophenyl acetic acid, 0.45mg/l benzylaminpurine (BAP), 0.43mg/l kinetin] 15g/l sucrose
SEIM: Somatic Embryogenic Initiation Medium as above but with 11.05mg/l 2,4,D
SEABA: Somatic Embryogenic Abscisic Acid Medium as above but only PGR 25mg/l abscisic acid

Cryopreservation
PVS2: Plant Vitrification Solution 2, (Sakai and Kobayashi, 1990) ethylene glycol, DMSO and glycerol

Tg: Glass transition temperature (Tg): temperature at which a liquid undergoes a phase transition to an amorphous glass. The point of change from a liquid to a glass (Tg) is detected as a deflection in heat flow on differential scanning calorimetry thermogram.
DSC: Differential scanning calorimetry – instrumentation that measures heat-flow during a preset (10°C/min) cool and warming cycle, between two pans (the sample tissue sealed in an aluminium pan, against the control reference pan) and shows the thermal difference (mWatts) plotted against time, or temperature, to produce a thermogram.
Programmable freezer: A programmable freezer is a commercial instrument, used to undertake controlled rate cooling of tissue. It comprises of a cooling chamber into which liquid nitrogen is supplied from a pressurized Dewar controlled by a solenoid valve, a chamber constructed to hold cryovials or straws and a computer software link which enables the programmer to specify a range of temperature rates, ramps and start and finish parameters.
Mr. Frosty ™: A 86*117mm (height*diameter) portable unit consisting of a high-density polyethylene container, containing 100% isopropanol that provides a cooling rate of –0.99°C/ min typically through placement for 1 hr in a fixed temperature freezer (-80°C) Nalgene ®.

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